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Re-defining the Concept of Model Species:
An Experimental Approach on a Range of Marine Animals

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ABSTRACT

Scientific research extensively uses model organisms to address biological, ecological and evolutionary issues. Research employs a limited variety of organisms, ranging from bacteria to complex metazoans, to answer scientific questions and investigate natural aspects. We define “Model Organisms” this small fraction of the biological biodiversity. Generally, they are a simplified tractable system used to study larger scientific questions and to answer complex problems. Researchers expect that results obtained through experiments on these MOs will be applicable to other, more complex, organisms, or to complex communities and ecosystems. Model organisms are sometimes a scarcely representative sample of the global physiological, biochemical and genetic biodiversity and the answers that they can provide could not be easily applicable and transferred to other species. This Ph.D. thesis aims at re-defining the concept of “model species” on an objective point of view. I analysed a standard range of common characteristics that can describe and rank selected model organisms on the basis of their unique features, advantages and disadvantages. I have taken into consideration various “practical” features, such as size and feeding, their reproduction and the optimization of the culture techniques; their use in some scientific fields, such as chemical ecology, stress responses and apoptosis, and the availability of molecular tools and sequenced genomes. A set of laboratory analyses was applied to test the power of selected species to answer our questions and an arbitrary score was assigned to each species for each parameter according to our results and to the data available in scientific literature. The information collected was used to objectively rank the considered model organisms, for improving future experimental approaches and indicate a possible strategy to choose adequate models for selected research.

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1 : INTRODUCTION

Our knowledge of natural sciences, such as heredity, development, genetics and physiology, is largely derived from studies on model organisms. Life evolved in a great variety of forms as well as morphological and physiological adaptations. Despite this great biodiversity, a common base of shared features can be identified studying a small group of life forms, carefully chosen taking into consideration their ability to respond to definite experimental studies (Müller and Grossniklaus, 2010). Research employing organisms, such as *Escherichia coli*, *Drosophila melanogaster* and *Mus musculus*, led to the discoveries of key mechanisms of heredity, gene regulation and metabolic disease respectively (Levy and Currie, 2015). This small fraction of Earth biodiversity (Fig. 1.1) typically used in scientific research, can be defined as Model Organisms (MOs) and their study forms the core of the biological knowledge (Hedges, 2002). In most general terms, MOs are non-human species widely studied to investigate natural aspects, hoping that results and theories obtained through experiments will be applicable to other organisms, usually more complex than the studied model. The term “Model organism” was used to indicate a simplified tractable system used to study larger scientific questions.



Fig. 1.1: Main model organisms commonly used in scientific investigations. (From Müller and Grossniklaus, 2010).

Model organisms are taken to represent a wide group of organisms beyond themselves, and serve as the basis for articulating processes thought to be shared across several other types of species (Leonelli and Ankeny, 2013). Researchers have focused their attention on these models to obtain an insight view into the principles that underlie various disciplines and it is undeniable that the use of few species has been critical in many fields of biology for centuries. The most quoted motto related to experimental practice is “For large

number of problems, there will be some animal of choice, or few such animals on which it can most conveniently be studied" (Krebs, 1975).

The first model organism in history can be considered the pea plant, carefully chosen by Gregor Mendel, an Augustinian friar and scientist of 19th century that investigated the universal questions of heredity (Orel, 1972). It is interesting to underline how much he focused on the characteristics of organisms used as model that they "must exhibit to avoid questionable results" (Stern and Sherwood, 1966). Which species proves to be advantageous for research, in other terms "find the right organism for the right job", is a function of several features. Indeed, the identification and standardization of new model organisms needs to take into consideration several key factors: phylogenetic position, biological suitability and experimental amenability to answer to specific scientific questions, according to techniques and practices available. In addition, "A model must be cheap and plentiful; be inexpensive to house; be straightforward to propagate; have short gestation periods that produce large numbers of offspring; be easy to manipulate in the lab; and boast a fairly small and (relatively) uncomplicated genome" (Bahls et al., 2003). A first phase is necessary to establish a long-term success model organism: a core set of experimental protocols is the pre-requisite to attract a critical mass of researchers.

The accumulated basic knowledge on individual species makes it easier to perform experiments without being tied down by technical difficulties. Each model organism has a threshold that, if surpassed, assures the self-perpetuation of the model due to its popularity, availability and the possibility to compare data with a rich research community. In a long-term point of view, the devising of a model organism can follow two methods. In fact, it is possible to discover some new fundamental characteristics that could not be anticipated initially, as is the case of the easy to transform *Arabidopsis thaliana* (Clough and Bent, 1998; Bechtold, 1993). Alternatively, the research community

may lose the interest in the development of a promising model due to the inadequacy of the organism to respond to new scientific questions, as in the case of *Cavia porcellus* (Guinea pig). Guinea pig has been extensively used since the 17th century, to investigate a wide range of natural phenomena such as anatomical structures, important theories such as Germ theory, facilitate the discovery of new compounds, for example Vitamin C, and to test vaccines such as diphtheria and cholera. The model lost the scientific appeal due to the difficult genetics (Crow, 2002) but also due to a long gestation period and relatively low birth rates that led to the substitution with mice and rats in laboratories all over the world. For these reasons, in some circumstances, the choice of a specific model organism, for a given category of experiment, can be considered accidental rather than carefully scheduled; in fact, organisms can be chosen since they were previously in use, laboratory were familiar with and/or because there was a huge research background about them. The choice of organisms to be used as models is part of the experimental design and a vision of the whole topic as well as knowledge of the available scientific techniques are needed. This choice should also reflect the level of the research question; for example, if the process of interest is evolutionarily conserved across all eukaryotes, then the yeast *Saccharomyces cerevisiae* can be considered a good model for investigations; if metabolic processes is restricted to a single taxa, such as vertebrate, zebrafish or mice should be considered more appropriate (Reed et al., 2017). In other cases, processes in chosen model organism can be simply too complex or there could be too many variables to obtain a certain scientific answer. Is this the case, for example, of researches on human diseases and on developing cures against them (Festing and Wilkinson, 2007; Rollin, 2007) where animal models completely failed to predict serious side effects as in the case of the immunomodulatory TGN1412, used to treat autoimmune rheumatoid arthritis, that caused a catastrophic trial in the United Kingdom. TGN1412 passed various animal trials,

including those in primates (Rosenthal, 2006), but caused in humans an unpredicted immune response and systemic organ dysfunction due to a severe allergic reaction. Despite the fact that the exact mechanism has not yet been understood, the unforeseen response of the immune system could be caused by the sterile environment, with insufficient exposure to pathogenic antigens, where the model organisms were raised (Hunter, 2008).

1.1 MODEL ORGANISMS

Several model organisms have been used in research in the last 20 years for specific purposes: sea urchin was extensively used in ecotoxicology and developmental biology (Falugi et al., 2012; Chiarelli et al., 2011; Semenova et al., 2006) and more recently, to study the effect of the ocean acidification (Kelly et al., 2013; Clark et al., 2009), Flatworms for studies on inheritance and regeneration (Gentile et al., 2011; Alvarado, 2004; Mitman and Fausto-Sterling, 1992), *Chlamydomonas reinhardtii* to investigate photosynthesis (Wykoff et al., 1998; Rochaix, 1995), *Dictyostelium discoideum* to study cellular communication (Strassmann et al., 2000; Devreotes, 1989), *Aplysia* spp. for neurobiological studies (Moroz et al., 2006; Siegelbaum et al., 1982; Frazier et al., 1967), Frogs to study lungs and respiration as well as endocrine investigations (West, 2013; El-Salhy et al., 1981; Pump, 1966).

One of the established and studied model organisms is the fruit fly *Drosophila melanogaster*, diffused as a key model in genetics and developmental biology. It was set in the first decades of the 20th century, thanks to the work of Thomas Hunt Morgan at the Columbia University and at Cal Tech. The importance of this model organism increased and regained importance in the research community when molecular genetics emerged in 1970s (Weber, 2008; Keller, 1996). Investigations on *Drosophila* were fundamental to support the chromosome theory, as mechanistic explanation of Mendel's rules, to identify and characterize the phenomenon of genetic linkage and to develop chromosomal mapping. *Drosophila* appeared as a very well-suited organism to be used in research due to its small size and short generation but also due to the possibility to maintain large population in a poorly equipped facility.

Within a few years, most flies were lab-reared and this led to the isolation and identification of new strains of interest that were bred and standardized in the lab. With

the development and establishment of this model, fruit fly genetics were intentionally modified to produce strains that were better suited for lab work, such as more viable, easier to score or simpler to cross strain. Furthermore, an array of experimental tools was developed, perfected and expanded. The increase of available techniques for manipulating cellular and molecular structures and the deep knowledge of virtually any gene, expressed by fruit fly, at each specific stage and location, amplified the affirmation of the model in many research fields. *Drosophila* is important as a model organism because it has been regarded as a basis for claims about other organisms: findings and results in fruit fly were applicable to a vast range of species, including both vertebrates and invertebrates, demonstrating, for example, that the basic mechanisms of Mendelian heredity are shared in all species with sexual reproduction (Morgan and Bridges, 1916; Morgan, 1915).

MOs are so convenient that a wide number of tools and resources were specifically developed for each considered species. Infrastructures such as databases and strain collections, molecular toolkits, as well as highly optimized procedures and methods were established. Differences were highlighted, in recent years, thanks to the availability of complete genome sequences from many model organisms, which has greatly facilitated comparisons among species and increased connections among research communities.

To explain how genome projects changed the model organism debate, once *Caenorhabditis elegans* genomes were available it made even less sense to work on everything else. Indeed, the gap between the few established and sequenced model organisms and all the other organisms used in research was so deep and the methodologies and resources were so different that there was an on-going linguistic modification in the concept of "Model organism". The term is nowadays used not in the

original meaning but, instead, in the sense of “a species or strain for which a huge number of tools and resources exist”.

Those few established model organisms have been proven to be not always the best help to answer all possible scientific question. In many fields of study, unsupported, old, model organisms were still a key point in experimental research. In the last years, the constant decrease in cost of genomic sequencing made it possible to sequence genomes and transcriptomes from a variety of organisms including old and emerging animal models. Small research groups are able to sequence under-supported model organisms and this has caused an explosion of interest in extending the set of model organisms applied for research. Genome and transcriptome sequencing allow researchers to apply new molecular approach, such as CRISPR, opening new fields of research (Table 1.1).

Kingdom	Phylum	Class	Number of genomes
Animalia	Annelida	Clitellata	1
		Polychaeta	1
	Arthropoda	Arachnida	5
		Branchiopoda	1
		Chilopoda	1
		Insecta	69
		Maxillopoda	1
	Chordata	Actinopterygii	1
		Amphibia	1
		Aves	11
		Mammalia	73
		Reptilia	6
		Leptocardii	1
	Tunicata	Appendicularia	2
		Ascidiacea	1
	Cnidaria	Anthozoa	2
		Cubozoa	1
		Hydrozoa	1
	Echinodermata	Asteroidea	1
		Echinoidea	2
	Hemichordata		1
	Mollusca	Bivalvia	1
		Gastropoda	2
	Placozoa		1
	Porifera	Demospongiae	1
	Platyhelminthes	Trematoda	3
		Turbellaria	1
	Nematoda	Secernentea	21
		Chromadorea	2
Fungi	Ascomycota		178
	Basidiomycota		48
	Other fungi		22
Rhizaria	Cercozoa	Chlorarachnea	1
Archaeplastida	Rhodophyta	Florideophyceae	1
		Cyanidiophyceae	2
Chromalveolata	Cryptophyta	Cryptophyceae	1
	Heterokontophyta	Bacillariophyceae	1
		Coscinodiscophyceae	2
		Eustigmatophyceae	2
		Oomycetes	12
Alveolata	Apicomplexa		20
	Ciliophora	Ciliata	1
		Spirotrichea	6
		Oligohymenophorea	1
	Perkinsozoa	Perkinsea	1
Excavata	Euglenozoa	Kinetoplastea	13
	Percolozoa	Heterolobosea	1
Choanoflagellata			2
Unikonta	Amoebozoa	Mycetozoa	2
	Metamonada	Parabasalia	1
Plantae	Chlorophyta	Chlorophyceae	2
		Trebouxiophyceae	1
		Trebouxiophyceae	1
		Prasinophyceae	4
	Metaphyta		62

Table 1.1: Number of sequenced eukaryotic genomes. Information obtained by National Center for Biotechnology Information, April 2013 (From Ellegren, 2014).

All fields advanced thanks to these new approaches, comprising evolutionary biology, in which the molecular analysis has explained phylogenetic relationships and divergence times among taxa (Hedges, 2002). In addition, new methods mean new answer to scientific questions and, as consequence, new animal models arose, such as Japanese

pufferfish or Turafugu (*Takifugu rubripes*) because of the unique characteristic of their very short genome rather than the capability to answer to experimental studies. Such types of organisms have been called “genomic” models by the research communities (Brenner et al., 1993).

Since the common concept of a model organism is changing due to technological advances in genome sequencing and editing, it appears hard to provide a complete list of model organisms. However, a list of canonical model organisms has been defined by the National Institute of Health (NIH) during the last decades. In the last two decades, a large number of new web resources emerged for a wide number of established and new model organisms (Tang et al., 2015). Many public web resources provide data for gene models, allelic variations, mutant phenotypes, anatomy function, expression patterns, gene interactions, human disease relevance etc., as in the case of WormBase, the web resource of the model organisms *Caenorhabditis elegans* (Harris et al., 2013), or the Zebrafish Information Network (ZFIN), the database for the model organisms zebrafish (Howe et al., 2012). Web resources are available for less established and studied model organisms too, as in the case of the colonial ascidians *Botryllus schlosseri* (Voskoboynik and Weissman, 2015; Voskoboynik et al., 2013).

1.2 AQUATIC MODEL ORGANISMS

Marine animals largely contributed to basic and applied biomedical researches, greatly improving human life, conducting to new scientific discoveries in many fields. The great biodiversity that the aquatic environments may offer, is considered a rich treasure that is waiting to be explored and the wide variety of specialized physiological, genetic and chemical adaptations are a great starting point for researchers to learn more about processes that cannot be studied in more complex organisms. Of the 33 animal phyla described, it is estimated that 32 are represented in aquatic environments, with 15 being exclusively marine (Margulis and Schwartz, 2000). Many of these animals grow and reproduce during all their life spans, without any apparent age-related decrease in functions, incidence of disease or increase in mortality rate due to aging. The high fecundity of aquatic organisms allows an easy breeding in facilities providing a sustainable and continuous supply of individuals for research and, in the meantime, avoiding any impact on the natural populations and ecosystems.

These organisms populate different environments, from poles, to coral reefs, to hydrothermal vents, with specialized features for their specific habitats. New applications of marine organisms to biomedical research are dependent on the diversity of these marine organisms that have adapted to different habitats over long-term evolution. However, this diversity is nowadays threatened by anthropic disturbances such as pollution and eutrophication, climate change and overfishing, on a global scale. The loss of marine species means a loss of potential food sources but also the loss of new molecules in biomedicine (Rosenthal and Grifo, 1997). Some taxa share a common ancestor with mammals, such as echinoderms, ascidians, hemichordates and cephalochordates, making them highly relevant models for investigation on human and vertebrate biology.

This is the case, for example, of the California purple sea urchin, *Strongylocentrotus purpuratus*, affirmed as model organism for a wide range of scientific investigations. It represents a powerful research model that has brought almost everything we know, for example, about chromosomal basis of development and the importance of maternal determinants and RNA in the offspring. *Strongylocentrotus purpuratus* is a small, spiny animals belonging to the class Echinoidea (Echinoderm phylum). The use of this model in research has some important advantages: a wide distribution, and availability of broodstock, in nature, inhabits the shallows and tide pools of ocean environments, a large numbers of synchronous developing embryos that can be obtained quickly and easily, a rapid embryonic development, a simple structure of transparent embryos and the possibility to perturb development using small molecules, such as chemical inhibitors and biosynthetic precursors (Angerer and Angerer, 2004). Numerous tools available, such as the pattern of various gene regulatory networks (Ben-Tabou de-Leon, 2016; Peter and Davidson, 2011; Levine and Davidson, 2005) as well as different molecular protocols (Cameron, 2014), and the recently obtained genome sequence (Cameron et al., 2009; Sodergren et al., 2006) provide a unique opportunity to address some crucial questions in developmental biology and cell cycle regulation as well as in molecular, evolutionary, cell biology and immune response field (Ettensohn and States, 2017). In addition, *Stongylocentrotus purpuratus* affirmed in last decade as model for climate change studies due to its sensibility to high temperature and low pH (Evans et al., 2015; Kelly et al., 2013; Stumpp et al., 2012).

As regards sea urchin, the use of *Paracentrotus lividus* as a model organism for scientific researches is increasing, in particular in European scientific community (Fig. 1.2). The development of new marine model organisms, useful as tool for eco-toxicological studies and for biotechnological application, was encouraged by the possibility to investigate

unexplored marine models' biology and physiology that could be associated with an innovative exploitation in marine biotechnology field, for the discovery of new pharmaceutical, nutraceuticals and cosmetic products as well as new aquaculture methods and technologies, biomaterials and bioenergy. *Paracentrotus lividus* shares with the phylogenetically related *S. purpuratus*, the abovementioned advantages as regard biology, reproduction, embryo development and some tools, such as gene regulation, although a complete genome sequence for this species is still not available.



Fig. 1.2: *Paracentrotus lividus* with the fan mussel *Pinna nobilis*, in a weak *Posidonia oceanica* meadow (Çınar & Bilecenoğlu, 2016).

The role of sea urchins as a model is such important due to the key evolutionary position that the phylum Echinodermata occupies with respect to vertebrates. Indeed, the echinoderms as well as their sister group, the hemichordates, are the only other deuterostome animals besides chordates (Fig. 1.3). As a matter of fact, sea urchins are more closely related to chordates, vertebrates and, as well, humans, than all the other

invertebrate models used in research (Simakov et al., 2015; Swalla and Smith, 2008; Winchell et al., 2002). The evolutionary relationship was confirmed by genome sequencing that revealed a range of genes shared with most vertebrate gene families (Sodergren et al., 2006). Therefore, researches focused on this phylum obtain results that can be translated, at least partially, on humans. For example, embryogenesis studies on sea urchins are of great interest on an evolutionary perspective because they may illuminate features of the developmental program of the last common ancestor of all deuterostomes.

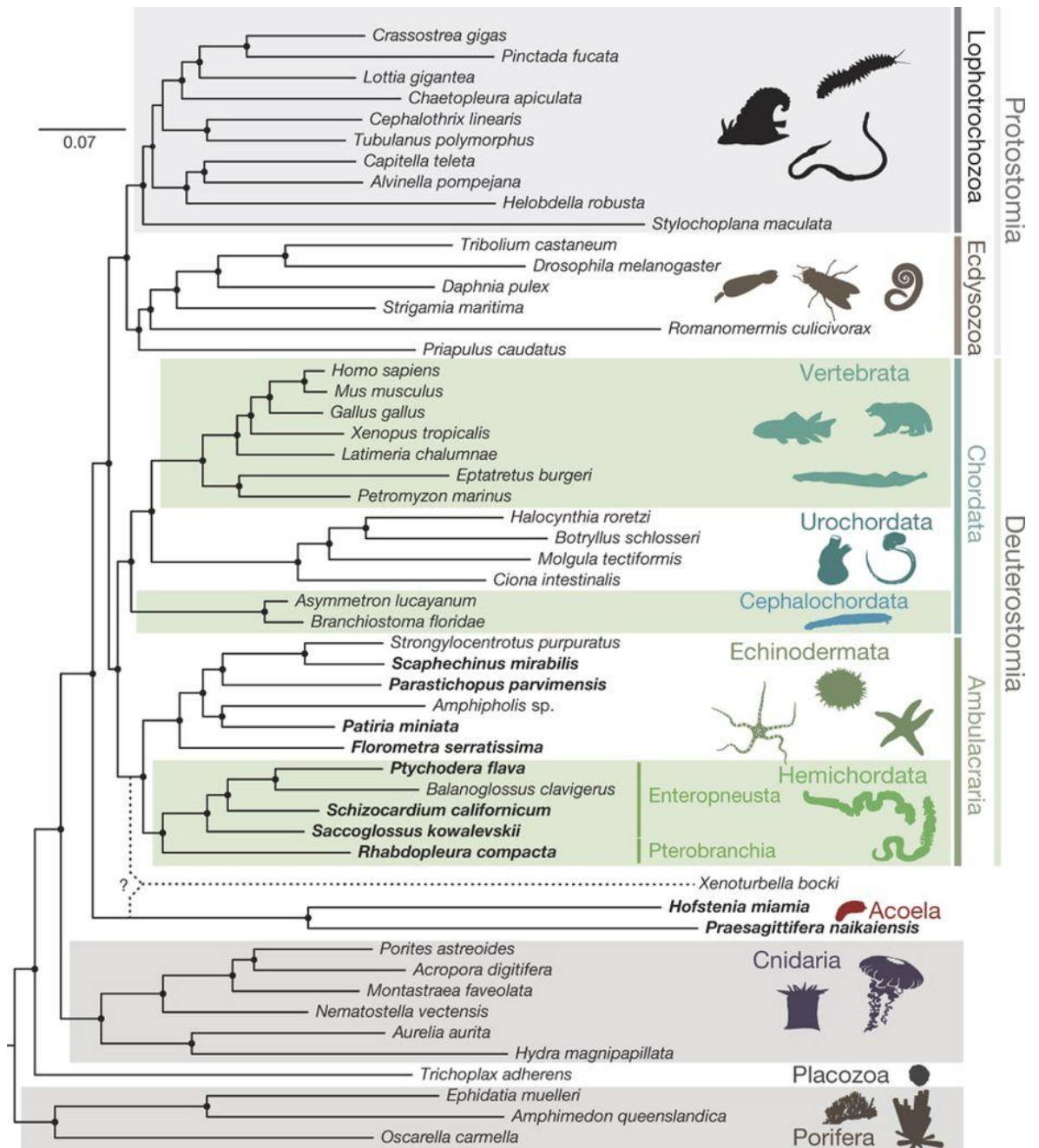


Fig. 1.3: Phylogenetic assignment of deuterostome within the metazoan tree (From Simakov et al., 2015).

The ethical impact of animal research on the public opinion should not be underestimated: investigations on non-mammalian species produce, generally, a greater social and ethical acceptance and, in the case of aquatic invertebrates, fewer regulation than that with vertebrates. Studies on sea star larvae that aided to form the foundation of

our understanding of cellular immunology, the investigations on the giant axon of squids, used to understand nerve conduction, on horseshoe crab eyes providing a model for photoreceptor physiology or on or sea hares that proved to be the right model organisms to study learning and memory (Bodnar, 2016). Studies on the mechanisms by which these organisms achieve unusual life histories may disclose how to protect against the destructive, and for now inevitable, process of aging and may led to new treatments to prevent or treat human aging and age-related degenerative disease.

This is the case of *Nothobranchius furzeri*, an annual fish from Africa that lives in seasonal ponds used as model organisms for aging research. The extreme environment in which they live conditioned their life cycle: the temporary existence of this habitat has obliged these organisms to compress their life in few months and to develop eggs that are resistant to desiccation (Fig. 1.4). Before the pond evaporates, males stimulate the females to release eggs that are then buried in the substrate. Eggs enter into a stage of diapause waiting for the arrival of the new rainy season when ponds form again. At the start of the new raining period, juveniles hatch and a new cycle begins (Cellerino et al., 2015; Dorn et al., 2014). Depending on the strain, the largest reported life expectancies range from 3 to 9 months (Valdesalici and Cellerino, 2003). As a vertebrate model, it has an extremely rapid life cycle that allow a wide range of investigation such as the study of drugs designed to impact vertebrate-specific genes or to assess their effects not only on longevity but, also, on age-related dysfunction of specific organ systems (Terzibasi et al., 2007).

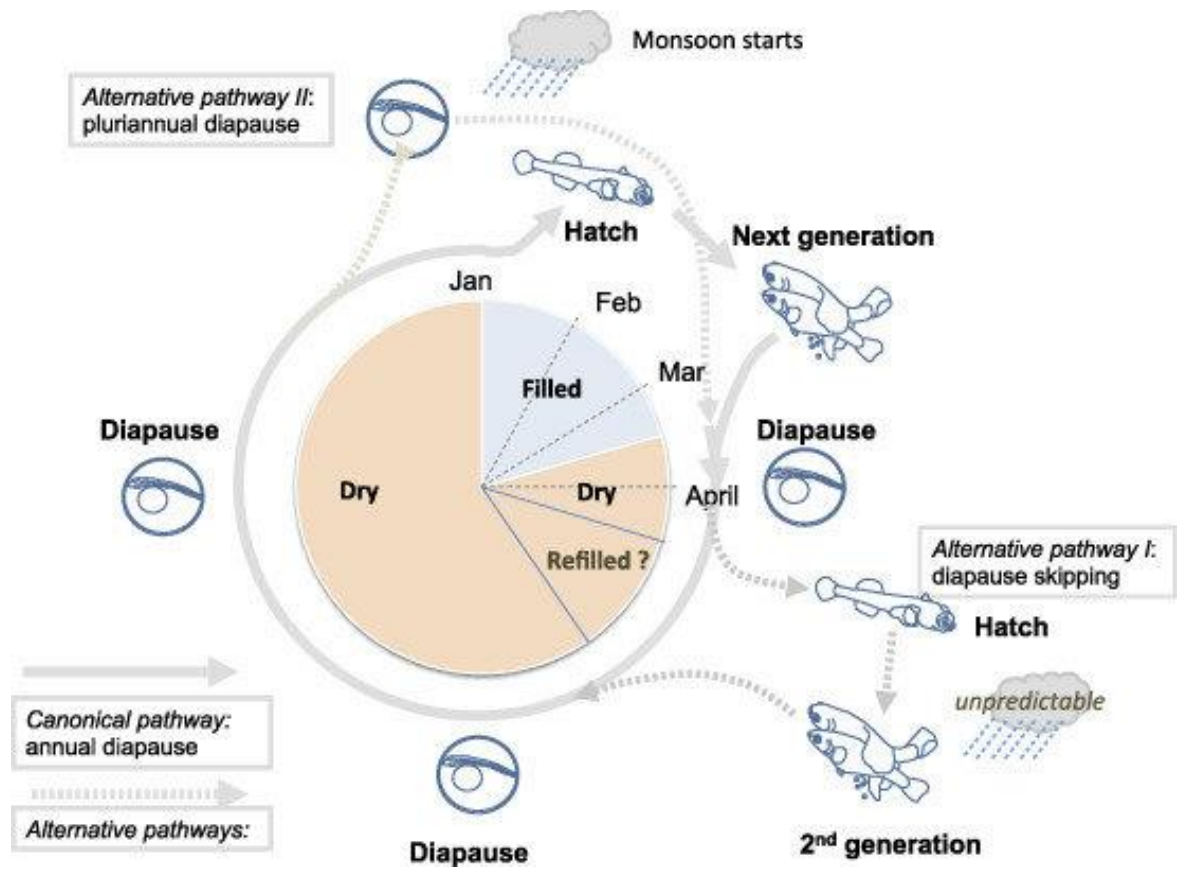


Fig. 1.4: Life cycle of the vertebrate model organism *Nothobranchius furzeri*, including alternative developmental pathways (From Cellerino et al., 2015).

The selection of the most appropriate organisms to perform specific research is a vital step in any research project and experimental design. “For most experimental biologists, life revolves around a handful of species: the mouse (*Mus musculus*), the nematode worm (*Caenorhabditis elegans*), the fruitfly (*Drosophila melanogaster*) and the thale cress (*Arabidopsis thaliana*). We assume that model organisms offer universal insights, and funding agencies largely support research on a shortlist of favoured species” (Bolker, 2012). Nevertheless, scientific investigations performed only on a few organisms, chosen as models, limit research to the answers that those organisms can provide. We cannot assume anymore that all the models are the same and that any model organism can be effectively used in single biological fields. In contrast, organisms used in experimental investigation do not necessarily have to be representative of species other than themselves (Leonelli and Ankeny, 2013). Scientific communities should think more critically to models, admitting the limits and the advantages of organisms used as target of scientific investigations and comparing them according to a more objective point of view. Model species are usually developed because of unique features such as the capability to answer specific questions, evolutionary constraints and availability of established breeding protocols and culture techniques; but the models can be merely used and selected because scientists are familiar with or they are easily accessible. Therefore, in many cases models are a much less random and representative sample of bio-diversity than we like to estimate. The recognising of obvious and less obvious limitations and disadvantages, exhibited by most standard models, does not involve we should repudiate model organisms and the use of them in scientific investigations. On the contrary, we should carefully estimate and take into account their limitations as an element of any research project and experimental design, for example, by analysing the role of a

gene in mouse strains with different genetic backgrounds. In PLoS Pathogens, a big debate arose about the use of animal models in the research of severe malaria (Craig et al., 2012). The debate culminated with the Hinxton Retreat meeting on “Animal Models for Research on Severe Malaria” (United Kingdom) where various researchers working on both animal models and humans discussed about research controversies. The first conclusion of the meeting was the need of a more intensive exchange of ideas among human research community and groups that works on various model organisms. In addition, a big debate about studies on animal models emerged, inferring that model organisms are not all the same, cannot be considered with the same scientific value and that studies on animal models cannot be always transferred to the human disease state. An introductory guide has been produced to help researchers to select the best invertebrate model host, in various investigation, in the context of fungal pathogenesis (Desalermos et al., 2012) outlining the questions that we should pose when choosing the organism that best fit our research theory because “no single model host can answer all scientific questions”.

1.4 AIMS

This research project aims at critically re-defining the concept of “model species” avoiding personal generalizations, by analysing a standard range of common characters that can describe and rank unique features, advantages and disadvantages of each species. In fact, model organisms (m.o.) are sometimes scarcely representative samples of biological diversity, being often chosen according to evolutionary constraints or personal confidence with peculiar taxonomical groups. The simplicity of rearing and the costs of maintenance versus the main physiological properties and the possibility to answer given research questions are issues taken into consideration. To this end, we screened a set of m.o. and observed their performances when applied to solve a common set of scientific questions. The objective of our research is to think critically about how scientific community choose and use animal models. In this way, we re-discuss the classical concept of m.o., based on the easy of rearing and the physiological plasticity, to be compared to the challenges afforded in the study of developmental biology and drug discovery. Due to the wide use of model organisms in all aspects of scientific research, this Ph.D. project can be approached from various points of view embracing many scientific fields. For these reasons, we decided to focalize the attention to the ecotoxicology, to apoptosis and to chemical ecology and infochemical fields, which can be considered as examples of many key areas of the scientific research.

We have identified a list of species, used as models, chosen among a broad set of marine organisms, and each species has been tested to identify unique features and performances. In particular, we used model species belonging to various taxa, especially those associated to seagrass complex ecosystems. A set of laboratory analysis has been applied to test the capacity of each species to answer to well-defined scientific questions.

A score will be assigned to each species based on literature and according to the results of our tests. Many parameters will be taken into considerations:

- **Size:** a score is assigned based on the size of each selected organism.
- **Management:** in this field we take in consideration the turnover of the species, the needs of highly specialized personnel, how much the organism is easy to feed.
- **Reproduction in laboratory:** we take into consideration whether the organism has a seasonal or continuative reproduction and the difficulties related to the breeding activities.
- **Rearing protocols set:** this score evaluate if there are or not specifically designed protocols or culture systems or if the organism can adapt to protocols and systems designed to culture other organisms.
- **Easy availability:** the score assigned in this field describe the availability and the easiness of collecting of each model in nature or in commercial market.
- **Stress response:** in this field we consider if each species is a good model to investigate stress response, in particular in the climate change field, drug mining and ecotoxicology.
- **Apoptosis:** a score is assigned to describe the capability of each model to be used in apoptosis researches. We consider, in this field, the production of apoptogenic compounds or the effects of apoptogenic compounds on selected model.
- **Chemical ecology and infochemicals:** is the model sufficient to investigate chemical, ecological interaction in nature? This field refers to the investigations on the chemical cues among organisms, behavioural responses and the effects of infochemicals and secondary metabolites on the organisms.

- **DNA/RNA:** are there molecular tools and protocols available for the considered organism? Is the DNA/RNA extraction easy to perform? How many genes are sequenced? We do not take into consideration ribosomal genes and cytochrome oxidase (COI).
- **Genome / Transcriptome:** is genome or transcriptome available for the selected species? Are genomes or transcriptomes of phylogenetically related species available?

Tests and analyses were designed in order to compile and fill a two-entrance table that can describe the value of each species for a particular field of use, its ease of breeding and any unique feature as well as the availability of genomic and molecular tools. All the information collected were used to impartially and objectively rank the considered m.o., also for improving future experimental approaches.

1.4.1 ECOTOXICOLOGY AND STRESS RESPONSES

Butler defined Ecotoxicology as “what is concerned with the toxic effects of chemical and physical agents on living organisms, especially populations and communities, within defined ecosystems, it includes the transfer pathways of those agents and their interactions with the environment.” (Walker et al., 2004).

The main goal of this field of study is to investigate the disturbance caused, both structural and functional, by chemical, biological and physical factors at short and long term. The toxicology investigations and the related stress in the target organisms can be inquired at various ecological levels, from cellular and molecular basis to the biocenosis, via the intermediary levels of organism and population. At the base of any ecotoxicological study there are the ecological processes that act on the species. The stability and recovery capacity of ecosystems depend on the characteristics of stress, such as the duration and frequency, as well as the history of the system that selected species with different resistance capability to stressors (Boudou and Ribeyre, 1997).

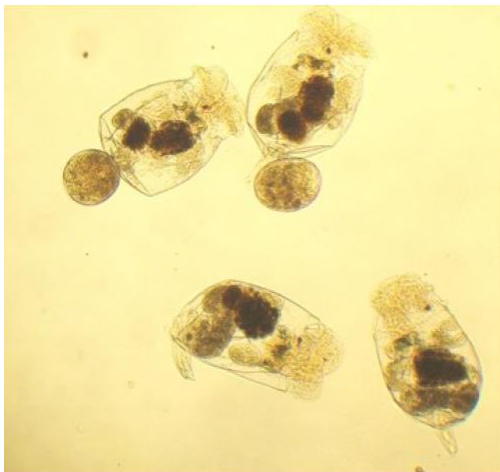
Since Ecotoxicology was developed by toxicologists with an interest in the environment, the basic principles of this field were those of toxicology: experimental tests, analysis of dose-effect relationship and estimation of responses to different concentrations. More recently, ecotoxicology focused the attention on toxic agents as stressors of biological systems. The stress ecology and the ecotoxicology merged to study the effects, often combined, of chemical, biological and physical stressors. The concept of stressor (an external factor), stress (an internal state caused by stressors) and the stress response (the cascade of physiological response triggered by stress) are at the base of the ecotoxicological investigations of stress. Stress responses can be studied at cellular,

biochemical and genetic levels but should be defined within the normal range of adaptation and ecological function of the target species: what can be considered an extremely stressful condition for a species, such as an increase of temperature or a very low concentration of oxygen, can be quite normal for other organisms that, due to evolutionary and ecological constraints, can be adapted to them. The stress can be considered as a condition evoked in an organism, by a certain number of stressors that bring the organism over the threshold of its ecological niche and physiological adaptation. Physiological studies are part of this multidisciplinary approach due to the importance of the stress-invoked specific responses, accompanied by counteracting mechanisms activated to maintain homeostasis. Consequently “stress can be defined in terms of a deviation from the state in a multidimensional space” (Straalen, 2003). To identify and inquire relevant and stress-specific processes, modern molecular and genetic tools can be applied to ecotoxicological studies to examine in detail key regulatory pathways and target genes. Reactions, observed at a molecular level, can be used to develop high sensitive bioanalytical tools to assess the hazard potential of chemical, biological or physical stressors (Schweigert et al., 2002).

Marine organisms, and more specifically marine invertebrates, have served as model system for marine ecotoxicology (Byrne et al., 2008; Carr et al., 2006; Coteur et al., 2003; Bay et al., 1993; Fig. 1.5). Bio-essays on echinoderm and mollusc gametes and embryos, due to their sensitivity to water chemistry, are fundamental tools to monitor toxicity of various pollutants. In fact, their gametes (externally fertilized) produce pelagic larvae that spend days to months in the water column (Byrne, 2011). Using established models as well as newly set aquatic organisms, researchers are now addressing the challenge to assess the impacts of stressors linked to global change, such as ocean warming, O.A. and hypercapnia (Byrne, 2011).



Fig. 1.5: Two common model organisms in Ecotoxicology: the brine shrimp *Artemia salina* (on the left) and the rotifer *Brachionus plicatilis* (on the right; From Agostino et al., 2016)



The sensitivity of marine organisms to ocean acidification vary among species and according to the life stages characterizing each species, challenging the ability of researchers to predict the consequences on species group and ecosystems (Byrne, 2010; Martinez, 2010; Kurihara, 2008). Research indicates that energy can be diverted away from crucial biological processes, such as growth and reproduction, towards compensatory responses (Beniash et al., 2010; Wood et al., 2008). Most studies in this field focused on a single stressor, e.g. warming or acidification, using species

predominantly adapted to shallower waters and intertidal model organisms, for developmental biology, ecotoxicology and aquaculture. Other taxa that have been a focus for climate change impacts include corals, crustaceans and polychaetes (Fig. 1.6).

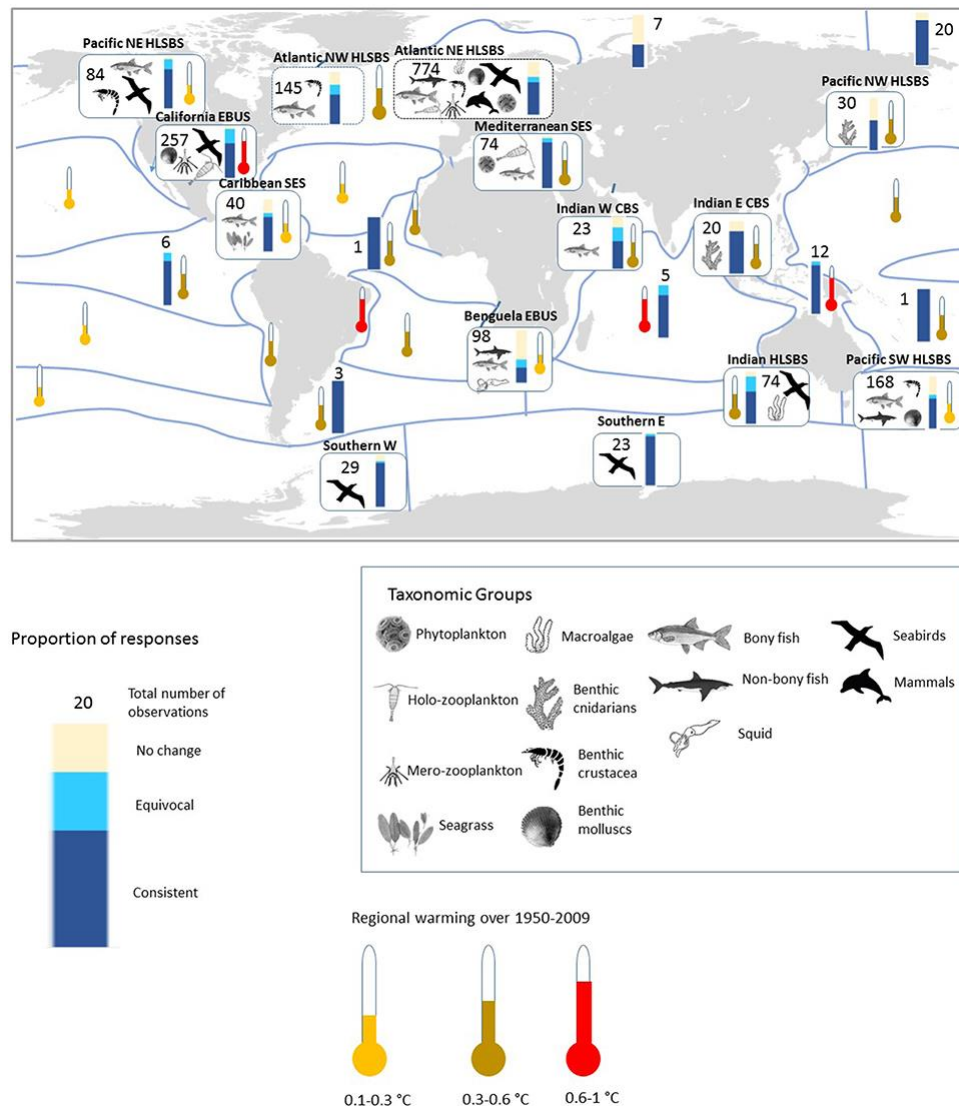


Fig. 1.6: The ecological impact of climate changes across ocean regions (From Poloczanska et al., 2016).

The sensitivity of marine crustaceans to ocean acidification is poorly understood, but it can be assessed by combining data from physiological and ecological studies. Marine species are exposed to higher risks, in particular those with limited physiological capacity to adjust their physiology according to environmental changes, because of poor iono- and

osmo-regulation capabilities to compensate acid-base disturbances. These species are typical of low-energy and stable environments where physical factors show little variation over temporal and spatial scales, such as cold-water species, due to limitation in the metabolic activities. Effects of long- and medium-term exposure to acidified seawater on the alteration of the calcification rates have been performed on species characterized by a commercial interest such as the prawns *Penaeus monodon* (Wickins, 1984), *Callinectes sapidus*, *Penaeus plebejus* and the lobster *Homarus americanus* (Arnold et al., 2009; Mangum et al., 1985). It has been demonstrated that in decapods calcification rates either remain the same or increase after a period of CO₂ exposure (Arnold et al., 2009; Wickins, 1984). On the contrary, effects of elevated seawater CO₂ on growth and reproductive indices have been detected in a wide number of commercial (Styf et al., 2013; Arnold et al., 2009; Wickins, 1984) and non-commercial crustacean species (Whiteley, 2011). Established crustacean models, such as the brine shrimp *Artemia* spp. and various *Daphnia* species were extensively used as models for studies of stress response and ecotoxicology (Boglino et al., 2012; Baruah et al., 2011; Jansen et al., 2011; Mbwambo et al., 2007; Han et al., 2006; Biesinger and Christensen, 1972) such as lethality tests to study the biological effects of cyanobacteria secondary metabolites in various environments (Lopes et al., 2010). An abundant scientific background permitted investigation on the physiological effects recorded in a high P-CO₂ environment, such as studies on acid-base regulation and on the ability of different species to counteract pH disturbances (Zheng et al., 2015; Locke, 1991).

Polychaetes constitute a high diversified and abundant group within benthic communities where they play a major functional role (Jumars et al., 2015; Hutchings, 1998). Furthermore, it represent a dominant part of the total abundance of the biological community at naturally acidified CO₂ vents (Garrard et al., 2014; Ricevuto et al., 2012,

2014; Calosi et al., 2013; Kroeker et al., 2011) and many studies used polychaetes to investigate potential responses to ocean acidification and the effects on benthic community composition and structure. In Ischia (Italy) CO₂ vents, studies (Calosi et al., 2013) focalized on few species, such as the Nereididae *Platynereis dumerilii*, the Opheliidae *Polyopthalmus pictus*, the Sabelidae *Amphiglena mediterranea* and the Syllidae *Syllis prolifera*, that are dominant in the most intense venting areas (Ricevuto et al., 2014; Kroeker et al., 2011) and associated with vegetated habits and with *P. oceanica* meadows (Giangrande et al., 2002, 2003; Gambi et al., 1992). In addition, polychaetes were extensively used to investigate physiological response to temperature shifts, pH decrease and salinity changes on tissue's regenerative capacity in *Diopatra neapolitana* (Pires et al., 2015), on the borrowing activity of *Nereis virens* (Widdicombe and Needham, 2007) and to investigate the combined effects of global changes with other stressors such as the presence of metal contaminants (Lewis et al., 2013, 2016; Campbell et al., 2014; Roberts et al., 2013).

Several benthic and planktonic invertebrates were also used to investigate indirect effects of O.A. In fact, besides the direct effects of O.A. on animal physiology (e.g. calcification and iono-regulation) above described, a totally new line of research indicates that indirect effects, as the disturbance of chemical signals, may buffer (Garrard et al., 2014) or influence in various ways the life of aquatic organisms (Poore et al., 2013). For example, it has been demonstrated (Zupo et al., 2016) that in acidified environments various benthic invertebrates do not recognize the presence of toxic compounds produced by benthic algae and that the water acidification influences the ability of planktonic copepods to recognize their algal foods (Maibam et al., 2015). In these studies the effect of O.A. is coupled to chemical ecology signals and the conclusions drawn thanks to the use of various animal models ranging from polychaetes to decapod crustaceans and molluscs

permits to identify the effects of O.A. on the structure of infochemicals (Zupo et al., 2015) and the effects on specific animal receptors by means of given chemotactic responses (Wyatt et al., 2014).

Apoptosis is an important form of programmed cell death (PCD) and it is one of the major evolutionary novelties characterizing multicellular organisms. Apoptosis can be found, at least in different forms, in all multicellular organisms (Koonin and Aravind, 2002), being described in mammals, insects, nematodes as well as in phylogenetically basal forms such as cnidarians, sponges and placozoans (Pernice et al., 2011; Lasi et al., 2010; Oberst et al., 2009; Srivastava et al., 2008; Pankow and Bamberger, 2007; Chipuk and Green, 2006; David et al., 2005; Seipp et al., 2001; Wiens et al., 2000). The molecular mechanisms of apoptosis were discovered in the nematode *Caenorhabditis elegans*, which subsequently became the first and the most popular model organism for these investigations (Hengartner, 1999; Liu and Hengartner, 1999). Homologs of *C. elegans* genes were identified in the fruitfly *Drosophila melanogaster* (Richardson and Kumar, 2002) and, in the same period, a larger apoptotic network was identified in humans and in the mouse (Manoharan et al., 2006; Koonin and Aravind, 2002; Meier and Finch, 2000). When investigating the evolution of apoptosis pathway, a key question is to understand how “old” is a protein and in which ancestral species it is likely to have originated (Zmasek et al., 2013). In the study of apoptosis, many model organisms were selected because of the availability of genomes and their “basal” phylogenetic positions, such as the demosponge *Amphimedon queenslandica* (Srivastava et al., 2010), the placozoan *Trichoplax adhaerens* (Srivastava et al., 2008), the hydra *Hydra magnipapillata* (Chapman et al., 2010) and the starlet sea anemone *Nematostella vectensis* (Putnam et al., 2007) in addition to deuterostomes such as the Amphioxus (*Branchiostoma floridae*) and purple sea urchin (*Strongylocentrotus purpuratus*; Zmasek et al., 2007).

Neurodegenerative diseases, cancer as well as several other pathologies are due to the deregulation of apoptosis: treating malignant cells through selective activation of

apoptosis, avoiding drug-resistance and side effects, has been recognized as a strategy in cancer therapy (Lowe and Lin, 2000) and it led to an increasing interest in finding new apoptotic compounds from nature. Marine environments are a rich source of molecules with biotechnological purposes: some molecules exhibiting apoptotic activity have overcome the clinical trials and have been introduced into the therapeutic protocols (Zelek et al., 2006). Diatoms were considered as fundamental species in in marine food webs, providing animals characterized by a rich content of fatty acids.

1.4.2.1 HIPPOLYTE INERMIS – COCCONEIS SPP. COEVOLUTION

Hippolyte inermis (Fig. 1.7) is a model organism for the investigation of apoptogenic compounds (Zupo et al., 2014; Zupo and Messina, 2007) and for studies on the mechanisms of sex reversal in decapods (Cobos et al., 2011; Zupo and Maibam, 2010).



Fig. 1.7: *Hippolyte inermis* female (From Zupo et al., 2014).

This is a protandric consecutive hermaphrodite shrimp (Yaldwyn, 1966; Veillet et al., 1963; Reverberi, 1950), distributed in west Atlantic sea, in the Sea of Marmara (D'Udekem D'Acoz, 1996) and in the Mediterranean seagrass meadows (D'Udekem D'Acoz, 1996; Gambi et al., 1992; Guillen Nieto, 1990). It is characterized by two different periods of recruitment (Zupo, 1994), the first in spring and yields offspring consisting of both males and females; the second in fall with offspring characterised by males that undergo sex reversal after the next spring recruitment (Veillet et al., 1963). *Hippolyte inermis* faces a unique sex reversal process that proceeds through the development of an ovary from undifferentiated germinal cells after the complete regression of the male gonad (Reverberi, 1950), lacking the intermediate stage of "ovotestis" (Cobos et al., 2005) that has been observed in other decapod crustaceans (Bauer and Holt, 1998). In addition to the sex reversal process observed in individuals aged about 1 year, a second mechanism may provide young and small females in natural population (Reverberi, 1950). Shrimps born in spring show early sex reversal leading to the production of small (from 6 to 7 mm of total size) females (Zupo, 1994). This process is directly related to the feeding on diatoms of genus *Cocconeis* (Fig. 1.8), a dominating component of the microphytic community associated to leaves of *P. oceanica* meadows, by early post-metamorphosed shrimps that cause the regression of the androgenic glands in juveniles.

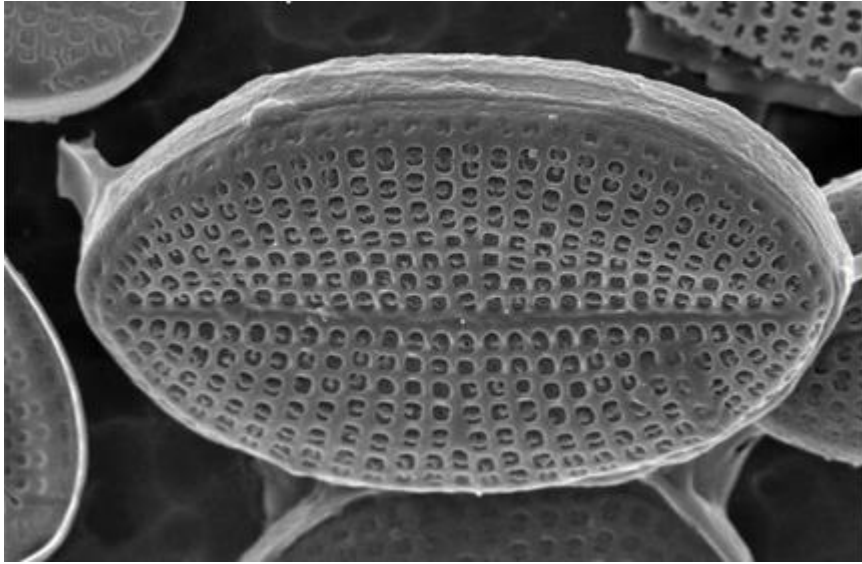


Fig. 1.8: *Cocconeis scutellum posidoniae*, a common epiphyte of *Posidonia oceanica* leaves (From Zupo et al., 2014).

In *H. inermis*, the ingestion of benthic diatoms (*Cocconeis* spp.) triggers the apoptotic early disruption of the androgenic gland that occurs in young post-larvae, in a very narrow time window (Zupo et al., 2006) due to the presence of diatom bioactive compounds specifically directed towards the androgenic gland. The process of sex reversal occurs during a single moult cycle (Zupo et al., 2007) and the action of diatom apoptotic compound(s) is species-specific, dose-dependent and extremely selective for the male gonad and the androgenic gland of *H. inermis* (Zupo and Messina, 2007). Diverse *Cocconeis* diatoms are also capable to trigger the sex reversal process but with various level of efficacy: *C. scutellum parva* and *C. posidoniae* are the species containing the highest quantity of active compound, while not strictly related diatoms, such as *Navicula* sp. and *Diploneis* sp. do not show evident effects (Zupo et al., 2007). Contemporarily, *Cocconeis* diatoms did not elicit any bioactivity on other crustaceans or on sea urchin embryos (Maibam et al., 2014; Rosen et al., 2013; Zupo and Messina, 2007). Tests on *H.*

inermis using toxic diatoms such as *Skeletonema costatum* did not produce any sex reversed young female and did not induce any mortality in postlarvae (Zupo et al., 2007). For these reasons, the apoptotic process targeted on the androgenic gland, is not due to a toxic effect but rather to a specific activity influencing shrimp physiology. The highly selective apoptogenic power demonstrated in the *Cocconeis-Hippolyte* case of study is important to develop new natural drugs useful for human anticancer therapies (Esmaeelian et al., 2013). Lipophilic fractions of the diatom *C. scutellum parva* have been demonstrated able to trigger apoptosis in human breast cancer cells (BT20 cells), *in vitro*, through the activation of caspase-8 and caspase-3 (Nicholson, 1999) without the activation of the intrinsic pathway, mediated by caspase-9 (Elmore, 2007). This proves that the activation promoted by the *Cocconeis* is not due to a general toxicity (Vine et al., 2007), but it is presumably caused by the highly-specific activation of ligands inducing apoptosis (Andrianasolo et al., 2008). Unfortunately, the chemical structure of the bioactive compound has not been elucidated yet and further investigations have been planned.

1.4.3 CHEMICAL ECOLOGY

The transfer of information among organisms, not necessarily of the same species, can exhibit various forms such as sight and hearing. Nevertheless, one of the oldest forms of communication in nature is the use of chemical signal molecules that can be considered ubiquitous (Thiyagarajan, 2010; Pohnert et al., 2007): from bacteria to algae to invertebrates to fishes, chemical signals play a fundamental role (Hardege, 1999; Pawlik, 1992) in the relationship among organisms in the environment (Ianora et al., 2011). If most organisms used in chemical ecology studies are terrestrial, in aqueous environment the chemical sense is predominant and many behaviours can be triggered via such chemical signals as mating partner and gametic release (Hardege et al., 1998), prey-predator interactions, detection of settlement sites (Rodriguez et al., 1993), induction of settlement (Zupo et al., submitted) and symbiosis (Murata et al., 1986). Aquatic organisms are part of a network of chemical information, where they exchange information “into which we have a very limited insight” (Brönmark and Hansson, 2012). In aquatic systems, chemical cues, called infochemicals, can be easily dispersed in a concentration sufficient to provoke a response in target organisms (Wisenden, 2000).

As term, infochemical can be defined as: “A chemical that, in the natural context, conveys information in an interaction between two individuals, evoking in the receiver a behavioural or physiological response that is adaptive to either one of the interactants or to both” (Dicke and Sabelis, 1988). If we consider the information itself an infochemical can be either a toxin or a nutrient as well as a volatile organic compound.

An infochemical can be a pheromone or an allelochemical: a pheromone is an infochemical that mediates an interaction between organisms of the same species and that can lead benefit to the source organism, to the target organism or to both organisms; on the contrary, an allelochemical is an infochemical able to mediate the

interaction of organisms that belong to different species. Among allelochemicals we can distinguish allomones, kairomones and synomones (Fig. 1.9) that evoke a behavioural or physiological response that is adaptively favourable, respectively, to the target organism, to the source organism or to either the interactants (Dicke and Sabelis, 1988).

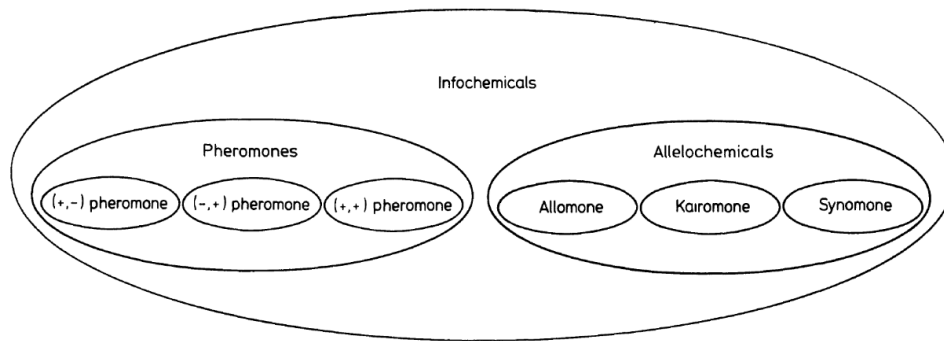


Fig. 1.9: Structure of infochemical terminology (From Dicke and Sabelis, 1988).

Marine organisms explore and interact with the environment primarily using olfactory senses, in order to collect information from their surrounding (Hay, 2009) and to take decisions about activities such as finding mates, feeding, habitat location, stabilize intraspecific hierarchal dominance and avoid predators (Breithaupt and Thiel, 2011; Peacor and Werner, 2001; Pawlik, 1992; Hay and Fenical, 1988). On an ecological point of view, chemical cues represent a web of information influencing the organisation of communities and the functioning of ecosystems (Pohnert et al., 2007; Hay and Kubanek, 2002).

Crustaceans are the most used models to study chemical intra-species communication: *Portunus sanguinolentus* (Christofferson, 1978), *Homarus americanus* (Cowan and Atema, 1990), *Pachygrapsus crassipes* (Kittredge, 1971), *Macropipus holsatus* (Eales, 1973),

Chinoecetes opilio (Bouchard et al., 1996), *Carcinus maenas* (Seifert, 1982) were extensively studied in order to detect and identify specific sex pheromones.

Nereidae are a family of polychaetes characterized by a spectacular mass spawning of the whole populations mediated by chemical signals emitted into seawater. Investigation on chemical signals in polychaetes were performed on two species, *Platynereis dumerilii* and *Nereis succinea*, that were chosen due to their reproductive behaviour, accessibility and easiness to culture in laboratory (Raible and Tessmar-Raible, 2014; Garcia-Alonso et al., 2013; Fischer and Dorresteijn, 2004; Hardege, 1999). The existence of pheromones in Nereidae family has been known for years (Lillie and Just, 1913): to demonstrate the presence of these compounds, biological assays were established using behavioural and electrophysiological methods (Boilly-Marer, 1974; Townsend, 1939). *Platynereis dumerilii* affirmed as one of the most important invertebrate model organisms in various scientific investigation from ecotoxicology (Yang and Zhang, 2013; Hutchinson et al., 1995) to ecology (Ricevuto et al., 2015; Giangrande et al., 2002) and evo-devo (Pfeifer et al., 2012; Steinmetz et al., 2011; Hui et al., 2009). If the chemical identity of many metabolites involved in reproduction or chemical defence are well known, we still know very little about the chemicals involved in, for example, assessment of predator threats, migration and plant animal interaction (Brönmark and Hansson, 2012). Nevertheless, using new transcriptomic approach, a rapidly advance in this research field was reached: *Danio rerio*, *Daphnia* spp. and *Aplysia* spp. were extensively used to investigate aquatic chemical ecology and to retrieve general information about how chemical senses, information, defence and communication function and what effects they have at community and ecosystem levels (Brönmark and Hansson, 2012). Pohnert et al. (2007) suggested that chemical structure of these defence metabolites can be determined using analytical

approaches even if it is a no easy task due to the fact that many infochemicals are rapidly degraded by bacteria in aquatic environment.

Actually, a boost in studies on marine algal defence chemistry has been achieved by pharmaceutical industry (Manilal et al., 2009; Cardozo et al., 2007; Ördög et al., 2004; Semesi, 1996; Michanek et al., 1979). Algae are a promising group to furnish novel biochemically active substances: freshwater and marine algae developed defence strategies to survive in competitive environments and show a significant level of chemical and metabolic diversity (Puglisi et al., 2004; Barros et al., 2001). Plant-animal communications encompass a wide range of biological processes. Studies on benthic faunal communities associated with seagrass meadows demonstrated that a) these ecosystems provide accommodations for a higher diversity and density of fauna than neighbouring unvegetated zones, b) this high biodiversity is stabilized by a complex and highly interconnected web of plant-plant, plant-animal, plant- physical environment, and animal-animal interactions (Orth et al., 1984) and c) chemical defences are among the most influencing chemical cues in these processes. Diatoms are an emerging group of model organisms and various species and strains are actively used as models for a number of investigations, such as *Thalassiosira pseudonana* used, for example, in biomineralisation (Sumper and Brunner, 2008), ecotoxicological (Erickson, 1972), evolutionary (Alverson et al., 2011), biotechnological (Wijffels and Barbosa, 2010; Pérez-Cabero et al., 2008; Lopez et al., 2005) and global change investigations (Hennon et al., 2015; Li and Campbell, 2013; Crawford et al., 2011).

Marine organisms are increasingly a major focus of natural products research efforts and are a rich source of novel and bioactive compounds. In this thesis, we used various organisms as source of bioactive compounds to perform test on our model organisms. These species may be considered from one side as a tool to test the performances of the selected model species, on the other as models in their turn, because they may be used for specific studies on the activation of specific genes in various environmental conditions.

- Cyanobacteria: are a prolific source of new compounds. They are photosynthetic bacteria with ubiquitous distribution ranging from marine to freshwater and terrestrial environments (Weissburg et al., 2012; Kulasooriya, 2011). They resemble both the features of bacteria and algae, exhibiting characters linking them to both groups (Stoyanov et al., 2014). High biodiversity is present in cyanobacteria groups and diverse forms can be observed ranging from unicellular to trichomes or colonial forms with branched or un-branched filaments and with or without heterocysts (Galhano et al., 2011). They can be found and can survive throughout the biosphere, ranging from Antarctic ice fields to thermal springs. Cyanobacteria are an interesting group of organisms for bio-technological purposes because they produce exclusive secondary metabolites (Dobretsov et al., 2011). Several bloom-forming species, growing in stagnant waters, are most often toxic to biota showing allelopathic activity on other cyanobacteria, algae and other organisms (Cheung et al., 2013; Jaiswal et al., 2005, 2008; Rodríguez-Meizoso et al., 2008). These allelopathic secondary metabolites are frequently released as secretions in the extracellular medium eliminating zooplankton and aquatic fauna, during bloom formation. Bio-activity and chemical structures of

many secondary metabolites of several cyanobacteria are available in literature. Furthermore, these prokaryotes are exceptional sources of compounds for pharmacological and biotechnological applications, such as foods, feeds, fuels, and pigments as fluorescent probes and in application aimed at combating pollution (Rastogi and Sinha, 2009; Gademann and Portmann, 2008). In addition, investigations focused on the screening of cyanobacterial secondary metabolites to search bioactive compounds that can be used in drug development cascades, as antidiabetic, antimicrobial, antifungal, antiviral, anti-inflammatory, anti-ulcerative and, especially, as anticancer medicines.

We isolated (Ruocco et al., in press) from the leaf stratum of the seagrass *Posidonia oceanica* and identified, in collaboration with other research groups, a free-living cyanobacterium showing 99% pairwise sequence identity with *Halomicronema metazoicum*, a species isolated for the first time as a symbiont of the sponge *Petrosia ficiformis* (Caroppo et al., 2012). Various strains of cyanobacteria were isolated from *Petrosia ficiformis* and aqueous extracts were tested to discover potential bioactive properties (Pagliara and Caroppo, 2011). Extracts obtained by cyanobacteria belonging to *Leptolyngbya* and *Synechococcus* genera (to which *Halomicronema metazoicum* belongs) exhibited cytolytic effect on human erythrocytes, toxic activity against nauplii of the model organisms *Artemia salina* and antimitotic activity against *Paracentrotus lividus* embryos. Due to these preliminary results and considering this strain as a potential source of novel bioactive compounds, we cultured this cyanobacterial strain to obtain enough biomass to perform bio-essays on various model organisms.

- Macroalgae: marine organisms use chemical cues to communicate among them and receive information produced, for example, by individuals of the same or different species, interpreting the intrinsic information to recognize trophic source or detect the presence of predators or dangers (Zupo and Maibam, 2011; Klaschka, 2009). Infochemicals can activate specie-specific reactions according to their role in the food webs (Zupo et al., 2016). Volatile organic compounds (VOCs), which can act as infochemicals, are fast spreading molecules that can be quickly transferred among organisms, even at medium and long distances from the source (Selander et al., 2016; Vet and Dicke, 1992; Dicke and Sabelis, 1988). This communication has evolved in various organisms, from bacteria to plants and animals, as biological apparatus to sense and process such information (Vos et al., 2006).

To investigate these fundamental aspects within the complex ecology of *P. oceanica* meadows and to test the use of potential model organisms in plant-animal interaction studies, we identified and isolated two species of diatoms (*Diploneis* sp. and *Cocconeis scutellum parva*) and a species of macroalgae (*Enteromorpha prolifera*), as epiphytes of *P. oceanica*, and we tested their infochemicals on two crustaceans (*Hippolyte inermis* and *Idotea balthica*; Fig.1.10) and two molluscs (*Alvania lineata* and *Rissoa italiensis*).



Fig. 1.10: Male (on the left) and female (on the right) individuals of *Idotea balthica* near a *Fucus* sp. meadow (From Haavisto, 2016).

In fact, previous studies demonstrate that micro and macroalgae, epiphytes of *P. oceanica*, produce volatile secondary metabolites playing the role of infochemicals for several invertebrates associated to the leaf stratum of seagrasses (Zupo et al., 2016). In addition, the behavioural responses of organisms to infochemicals might be shifted and inverted in acidified conditions (Zupo et al., 2016). For these reasons, we cultured our three sources of infochemicals at normal and acidified pH and we tested them at both pH conditions.

- Diatoms: are an important component of marine food webs (Steele, 1974) and they represent a fundamental constituent of the marine food source in planktonic and benthonic environments (Mazzella and Russo, 1989). Diatoms are characterized by a strong mechanical defences (Hamm et al., 2003) that protect them, at least partially, from the activities of small grazers (Sunda and Shertzer, 2012). At the same time, diatoms are vulnerable to grazing activities of larger

herbivores that are able to crush the silica frustules. To defend against these larger herbivores, diatoms evolved a complex set of deterrent compounds (Leflaive and Ten-Hage, 2007, 2009), such as toxic oxylipins including polyunsaturated aldehydes (PUAs) and oxo-acids (Ruocco et al., 2016; Fontana et al., 2007; Wichard et al., 2005). Oxylipins are wound-activated compounds (WACs) and are released after cell damage through an enzyme cascade that rapidly oxidizes polyunsaturated fatty acids (PUFAs), that are normal constituents of the cell membrane, to toxic end products (D'Ippolito et al., 2003; Pohnert, 2000). Oxylipins are not the only wound activated compounds produced by diatoms (Pohnert, 2000). They produce other deterrent compounds according to seasonal variations, strains, light irradiance and other environmental factors (Taylor et al., 2009). The defence compounds produced by diatoms have a strong effects on grazers, for example reducing copepod hatching success and egg production on diatom-dominated diets through the block of embryogenesis (Miralto et al., 1999) but additional mechanisms are proposed (Poulet et al., 2007). Various co-evolutionary processes may have modified the plant-animal relationships and many invertebrates have developed the ability to detoxify defence compounds and metabolites (Taylor et al., 2012; Lauritano et al., 2011) produced by algae. This co-evolutionary processes are particularly evident in those invertebrates feeding on diatoms (Zupo and Messina, 2007) as in the case of *Hippolyte inermis* (Maibam et al., 2014; Zupo et al., 2014; Zupo and Maibam, 2011). *Cocconeis scutellum parva* and *Cocconeis scutellum posidoniae* were cultured in axenic conditions in laboratory to assure sufficient biomass to investigate changes in the production of apoptogenic compound by *C. scutellum parva* when cultured at two

pH levels and testing it on the model organisms *H. inermis*, the only known “biological sensor” able to track the presence of this apoptogenic compound.

2 : CULTURE OF AQUATIC MODEL ORGANISMS

2.1 AUTOMATED CULTURE OF AQUATIC MODEL ORGANISMS: SHRIMP LARVAE HUSBANDRY FOR THE NEEDS OF RESEARCH AND AQUACULTURE

The work presented in this chapter has been published previously:

Mutalipassi, M., Di Natale, M., Mazzella, V., & Zupo, V. (2018). Automated culture of aquatic model organisms: Shrimp larvae husbandry for the needs of research and aquaculture. *Animal*, 12(1), 155–163.

2.1.1 ABSTRACT

Modern research makes frequent use of animal models, i.e., organisms raised and bred experimentally in order to help the understanding of biological and chemical processes affecting organisms or whole environments. The development of flexible, reprogrammable and modular systems that may help the automatic production of “not-easy-to-keep” species is important for scientific purposes and for such aquaculture needs as the production of alive foods, the culture of small larvae and the test of new culture procedures. For this reason, we planned and built a programmable experimental system adaptable to the culture of various aquatic organisms, at different developmental stages. The system is based on culture cylinders contained into operational tanks connected to water conditioning tanks. A programmable Central Processor Unit controls the operations, i.e., water changes, temperature, light irradiance, the opening and closure of valves for the discharge of unused foods, water circulation and filtration and disinfection systems, according to the information received by various probes. Various devices may be set to modify water circulation and water changes to fulfil the needs of given organisms, to avoid damage of delicate structures, improve feeding performances and reduce the

risk of movements over the water surface. The results obtained indicate that the system is effective in the production of shrimp larvae, being able to produce *Hippolyte inermis* postlarvae with low mortality as compared to the Standard Operation Procedures followed by human operators. Therefore, the patented prototype described in the present study is a possible solution to automate and simplify the rearing of small invertebrates in the laboratory and in production plants.

2.1.2 INTRODUCTION

Various organisms are widely used in biological research in order to understand the functions of life forms (Murthy and Ram, 2015). Despite the diversity of life forms, cellular and molecular processes, as well as vital functions are sometimes conserved and they can be -at least partially- compared (Griggio et al., 2014). In addition, several organisms are needed for aquaculture purposes, as live foods to be used only in given phases of the production process, or as main targets of aquaculture practices, during the larval phases (Buttino et al., 2012; Calado et al., 2003). In this case, small programmable devices may dramatically reduce production costs and the need for personnel and fixed setups (Acierno and Zonno, 2010). Programmable devices may consistently and cost-effectively repeat standard operations with higher precision, permitting to re-direct the personnel resource to other indispensable tasks.

Common model organisms may not be used for any purpose: “choosing the right organism for one’s research is as important as finding the right problems to work on” (Brenner, 2003). The increasing use of aquatic models is both an opportunity and a challenge for science and aquaculture: studies ranging from diet and culturing density to the management of facility spaces, up to the animal physiology with the aim of optimizing protocols and procedures, make them simpler, cheaper and more efficient as a fundamental step for the success of any model species (Zupo et al., 2011). While adult specimens, collected in the field, are usually prone to captivity problems (diseases, stress), the management of conditioned organisms implies a wide spectrum of issues, e.g., definition of long-term complete diets, set-up of high-density re-circulating systems, reduction of water-volume/organism ratio.

In some cases, model organisms are characterized by remarkable complexity and may be difficult to breed and culture but they are the only targets for particular compounds or for the study of specific mechanisms (Howe et al., 2013).

For example, *Hippolyte inermis* (Leach), a marine decapod crustacean living in the seagrass *Posidonia oceanica* (L. Delile) meadows, is important as a model organism, since it undergoes a peculiar process of protandric sex reversal (Zupo et al., 2008; Reverberi, 1950) due to apoptosis of the Androgenic Gland. Unfortunately, bioassays on its postlarvae, needed to test the effect of experimental apoptogenic compounds (Nappo et al., 2012), involve complex culture practices (Zupo, 2001). The culture of *H. inermis* larvae, in fact, involves specific operational work to collect and manage ovigerous females, perform sterile water changes and daily check larvae, avoid contamination by potential pathogens, and distribute alive foods.

Therefore, it is important to develop flexible, programmable and modular culture systems facilitating the automatic production of demanding species, both for scientific and aquaculture purposes. In fact, dedicated culture systems should satisfy the physiological needs of target organisms (temperature, dissolved oxygen, pH and salinity), reduce the abundance of decaying organic matter and the concentration of pollutants (e.g., nitrogen compounds), avoid the introduction of pathogens and reduce the stress that might alter behavioural and physiological patterns.

For this purpose, we devised and registered (Patent W02016166696A1) a programmable experimental system for the automatic culture of a range of aquatic organisms characterized by different features, at various developmental stages. The patented culture system is compact and it can be used for research or aquaculture purposes; it is modular and contains a Central Control Unit (CCU), programmable in Ladder language to modify culture protocols and meet the needs of a range of species. We tested the

automatic culture system on *Hippolyte inermis*, to verify its productivity and the performances in the culture of larvae of this delicate and demanding species.

2.1.3 MATERIALS AND METHODS

2.1.3.1 GENERAL LAYOUT

The larval culture equipment consists of four cylindrical units immersed into two tanks (Fig. 2.1).

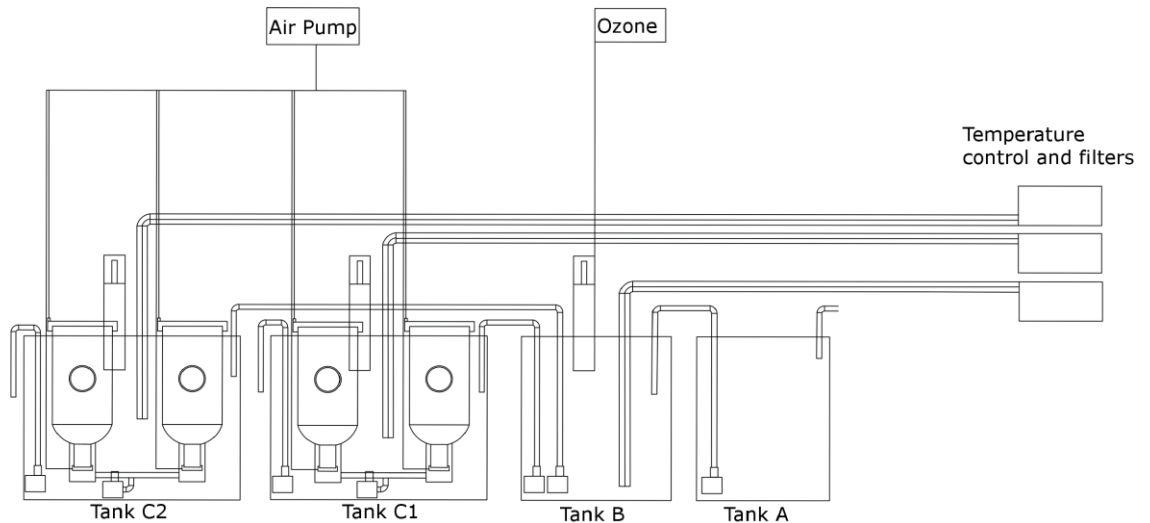


Fig. 2.1: Overview of the automated culture equipment. The boxes on the left indicate chillers and filters and they are connected to the corresponding tanks. The first tank on the right (tank A), contains seawater for storage. The pump inside this tank moves water to the tank B, where it is sterilized and filtered. The skimmer inside the tank B is connected to an ozone generator. The two pumps perform the connection between the tank B and the two treatment tanks C1 and C2. Two larval units are disposed in the tank C1 and used for the low density treatments and two in the tank C2 and used for the high-density treatments. A pump is connected by means of an aspiration tube to the bottom of each couple of cylinders. Air pumps allowed air insufflation in the treatment tanks, cylinders and under the nets located at the bottom of each cylinder.

Each tank is equipped with a canister filter (Eheim Classic 350) loaded with activated carbon and perlite wool, a chiller (Teco Tr-5 160 w or Teco Tr-10 260 w), a heater (Askoll Tronic 200 w) and a protein skimmer (Ferplast Bluskimmer 550, 350 litters per hour air flow). An ozone generator (Sander Certizon) is also present in the tank B and it can be operated to assure disinfected water for delicate organisms. Two water level sensors are located in each tank. The first one reads the maximum level and it is located 200 mm under the tank edge; the second one reads the minimum level and it is located 100 mm under the edge. The first tank (A) is 400 x 500 x 500 mm (h); it is a storage that receives continuously pumped water and permits a partial sedimentation of particulate matter. The second tank (B), is 450 x 500 x 500 (h) mm. Herein, the water received from tank A is sterilized and filtered. Two water pumps (Askoll Biodinamics 4, 620 litters per hour water flow), located in tank B, move the water to the tanks C1 and C2, when operated. These last tanks are 650 x 500 x 500 (h) mm and are equipped with water pumps (Askoll Biodinamics 4), connected to the drainage. Two cylindrical larval rearing units (described below) are submerged in each of the tanks C1 and C2. Only indirect natural light was shed during our experiments. However, in case of need, the system may be served by one or two fluorescent lights located over the tanks and controlled by the CCU.

2.1.3.2 LARVAL REARING UNITS

Each larval unit is made of a cylinder, a base, a dripper and a porous stone with its holder. The cylinder is a modification of a standard tronco-conical larval rearing unit commonly used in aquaculture practices, measuring 350 millimetres in height and 180 millimetres in diameter, with a total volume of ca. 10 litres. They are equipped with two holes: the first one, placed laterally, is 36 mm in diameter and it is provided with a 50 µm net; the second, located on the bottom, is 50 mm in diameter and is provided with a 250 µm net. The base and the cylinder holder work synergistically to allow the collection of a large air

bubble acting as a stopper, under the 250 µm net. The bubble can be removed by a pump (Sicce Syncra 1.0), located at the base and operated by the CCU. The air, blown into the lower part of the cylinder and inside the cylinder, comes from an air pump (Schego Prima 100 litres / hour), operated as well by the CCU.

The dripper is made of two PVC parts, joined to make a single ring and creating an interspace equipped with 16 holes (1 mm diameter) in the outer face. This latter is connected to a water pump (Sicce Syncra 1.5). When activated, it drives water under pressure in the ring cavity and through the holes, along the cylinder walls, creating gentle flows on the cylinder wall, to avoid larvae to remain adherent to the walls during water changes.

2.1.3.3 AUTOMATION

The automation of the larval culture equipment is assured by a CCU, Zelio logic Programmable Logic Controller (model SRC2261BD Schneider Electric) programmed in FBD language. Level sensors are connected via 0-10 volt digital inputs and technical accessories are connected via relay outputs. During the “stationary operation” phase, the system is programmed to activate lights, pumps, skimmers, filters, chillers, heaters and the two aforementioned aerators: the first connected to the porous stones, the second to the bases of the cylinders. According to the software developed for this equipment, the second phase begins at 08.00 a.m.: the water pumps placed at the base of culture cylinders are switched on for 5 hours. The pumps connected with the drippers are switched on at 08.00 a.m. up to 12.00, starting to pump water into the cylinders. Two pumps (Askoll Biodinamics 4), one for each culture tank, are switched on at 12.01 p.m. and they pump water to the drainage. Two pumps (Askoll Biodinamics 4) controlled by liquid level floats are subsequently switched on after 30 seconds, pumping water from tank B to tank C1 and C2 respectively. In order to minimize the mixing of clean water and

used water, the drainage pumps are positioned on the left side, whereas clean water inlet pipes are placed on the right side of each tank. For the same reason, inlet pipes are modified in order to create a non-turbulent laminar flow into the tanks C1 and C2. When tank B is empty, a liquid level float signals to CCU to switch off all the water change pumps. A Boolean logic subroutine activates the refilling and disinfection of tank B with ozone, and then reactivates the “stationary operation” phase. The ozonization process consists of the switching on of the skimmer and the ozonizer in tank B. After 5 hours the ozonizer is turned off while the skimmer is kept working in order to accelerate the removal of residual, potentially toxic, ozone.

2.1.3.4 COLLECTION OF OVIGEROUS FEMALES

Individuals of *Hippolyte inermis* were collected in a *Posidonia oceanica* meadow off Lacco Ameno d’Ischia (Zupo, 1994) and sorted on board the SZN vessel Phoenicia in May 2013. Samples were examined in the laboratory under a Leica MZ6 stereomicroscope and divided in 1500 mL conical flasks. Larvae produced by ovigerous females were collected, pooled and divided in sixteen replicates each cultured in a 800 mL conical flask according to a standard culture procedure described by Zupo and Messina (2007). Cultures were repeated in automatic culture system, in two replicates for each of two culture densities, i.e., low density (0.1 larvae.mL⁻¹ corresponding to 370 hatched larvae), and high density (0.15 larvae.mL⁻¹ corresponding to 550 hatched larvae). The experiment was repeated twice to obtain four replicates of each culture density. Larvae were fed on *Brachionus plicatilis* (5 ind.mL⁻¹) along with *Artemia salina* nauplii (5 ind.mL⁻¹) for 7 days. *Artemia salina* metanauplii enriched with AlgaMac 2000 (BioMarine Inc.) were used in replacement of the previous food from the 8th day onward.

2.1.3.5 WATER ANALYSES AND LARVAL DENSITY MEASURES

Measurements of chemical, physical and biological parameters were carried out daily in the culture equipment. The main chemical features of the water were measured: Redox potential (using a Martini Instruments ORP57WP portable ORP meter), pH (using a Mettler Toledo S62 pH portable tester) and concentration of nitrogen compounds and phosphates (using a Hach DR/2010 spectrophotometer and pre-prepared kits). The temperature was daily measured by means of a digital probe (TFA SDT8A). Larval density in control treatments was daily evaluated by counting all the larvae present in each conical flask after collecting them, individually, by means of a Pasteur pipette. Larval density in the test equipment was evaluated every day in three 200 mL samples of the culture medium, collected in each cylinder after continuous and intense agitation of the medium, to reduce patchiness.

2.1.3.6 DATA TREATMENT

Data collected were organized into datasets, as indicated in Table 2.1.

			NR of	
	Parameter(s)	Frequency	Samples	Replicates
Dataset 1	pH	Daily	22	4
Dataset 2	Temperature	Daily	22	4
Dataset 3	Concentration of inorganic pollutants	Daily (nitrite, phosphate), or twice a day (ammonium)	21	7
Dataset 4	Concentration of compounds in cylinders	Daily (nitrite, phosphate), or twice a day (ammonium)	33	6
Dataset 5	Survival in control cultures	Daily	18	16
Dataset 6	Survival in automatic cultures	3 x day	33	3
Dataset 7	Redox (tank A; tank B)	3 x day	21	2

Table 2.1: Experimental plan and dataset arrangement. For each measured parameter, the frequency of recordings and the number of samples and replicates considered is given.

Datasets were filed into MS Excel 2010 spreadsheets. Survival rates in the automatic larval culture were evaluated by plotting the average number of larvae present each day in each replicate. Prior to collect replicate samples and during the collection, the water was strongly agitated in each cylinder by means of a 50 mL plastic stripette. The survival rates were calculated as:

$$\left(\frac{ANL \cdot 18}{INL}\right) \cdot 100$$

Where:

ANL = average number of larvae contained in 200 mL samples

INL= initial number of larvae/mL

Wilcoxon matched-pairs test was applied to evaluate the significance of differences of ammonia, nitrite and phosphate concentrations between tanks C1 and C2 and among different replicates and treatments. Survival rates among low density cylinders, high density cylinders and control cultures were analysed by Mann Whitney test.

2.1.4 RESULTS

2.1.4.1 AUTOMATIC LARVAL CULTURE

The temperature measured in the tank A (water supply and storage) of the experimental equipment increased from 15.8 °C to 22.0°C during 21 days of test (average value of temperature was 17.8 ± 1.8 °C), due to seasonal natural variations of the seawater pumped in. Differently, the temperature was stable in tanks B (sterilization tank), C1 and C2 (culture tanks) during the experiment (tank B, 18.5°C, ± 0.1 ; tank C1, 18.5°C, ± 0.1 ; tank C2, 18.5 °C, ± 0.1) due to the thermoregulation applied. Similarly, the pH was stable during the experiment, as indicated by small standard deviations exhibited in daily measures in all tanks (tank A, pH 8.29, ± 0.06 ; tank B, pH 8.28, ± 0.05 ; tank C1, pH 8.28, ± 0.05 ; tank C2, pH 8.29, ± 0.04). Redox potential was checked three times a day: before, during and after the planned automated ozonation activities, to guarantee the disinfection and the removal of residual ozone. The maximum concentrations of ammonium, nitrites and phosphates in tank A were 0.05, 0.016 and 0.16 mg/L, respectively (Fig. 2.2A).

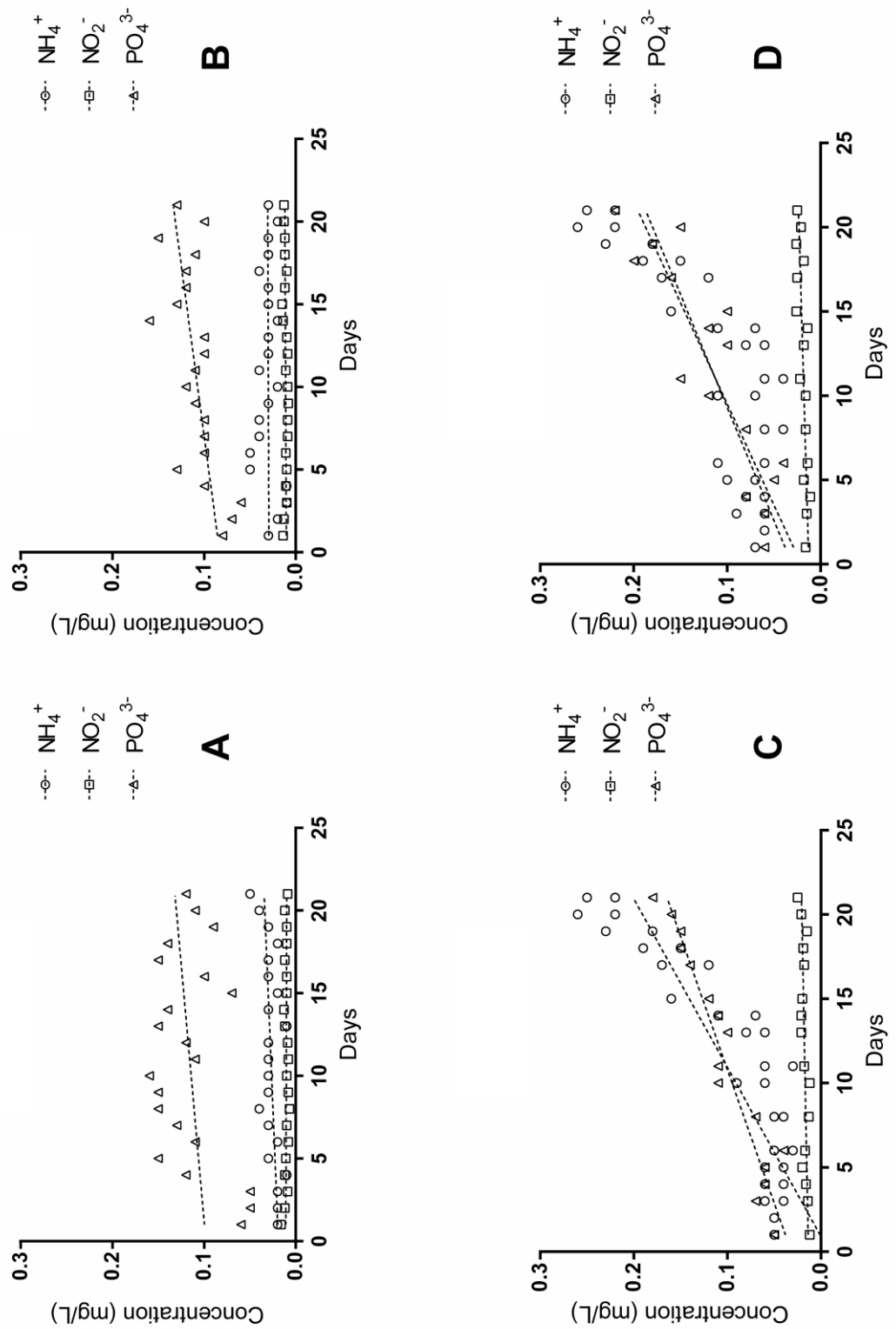


Fig. 2.2: Concentration and trends of NH_4^+ , NO_2^- and PO_4^{3-} in the tanks A (A), B (B), C1 (C) and C2 (D).

They were 0.05, 0.015 and 0.16 mg/L in tank B, respectively (Fig. 2.2B). Tanks C1 and C2 showed a linear increase of ammonia, nitrite and phosphate concentrations during the experiment. Maximum concentrations of nitrogen compounds in tanks C1 and C2 (Fig. 2.2C and 2.2B) were: 0.26 mg/L of ammonia at the 21st experimental day in both tanks; 0.025 mg/L of nitrite at the 21st day in the tank C1 and 0.026 mg/L of nitrite at the 15th and 19th day in the tank C2; 0.18 mg/L. of phosphate in tank C1 and 0.22 mg/L in tank C2 at the 21st day of experiment. Significant differences in the ammonium concentrations were observed between the tanks C1 and C2 (Wilcoxon matched-pairs test, $p < 0.0001$). On the contrary no significant differences were observed in nitrite (Wilcoxon matched-pairs test, $p > 0.05$) and phosphate (Wilcoxon matched-pairs test, $p > 0.05$) concentrations in the same tanks. Ammonium concentration (Fig. 2.3) in low density replicates (Cylinders 1 and 2) reached a maximum of 0.91 mg/L at the 20th day in Cylinder 1 and 1.10 mg/L at the 19th day in Cylinder 2; ammonium concentration reached higher values in replicates at high density (Cylinders 3 and 4), with a maximum concentration of 1.44 mg/L in Cylinder 3 (20th day of experiment) and 1.30 in Cylinder 4 (19th day of experiment).

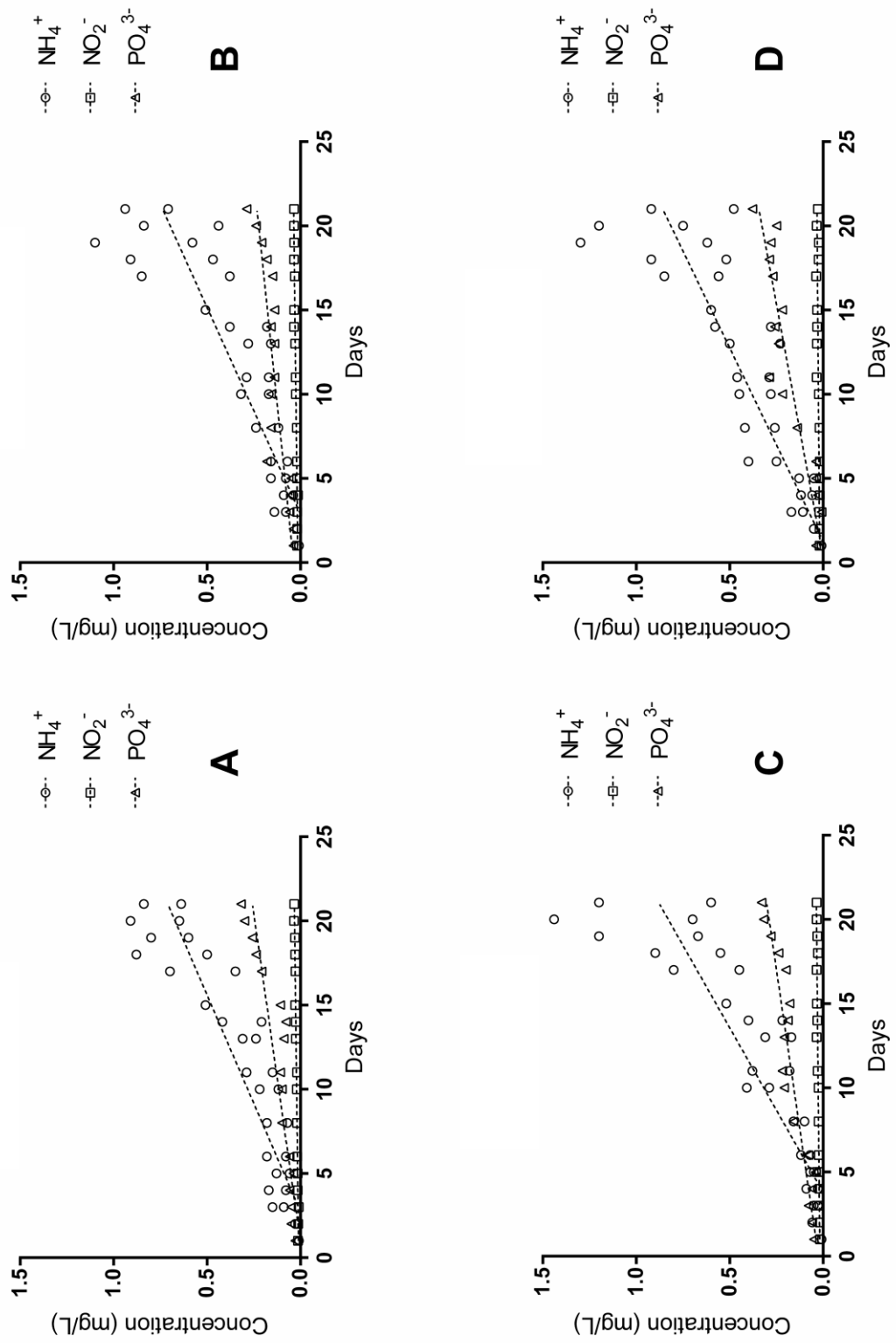


Fig. 2.3: Concentrations and trends of NH_4^+ , NO_2^- and PO_4^{3-} in low density Cylinders 1 (A) and 2 (B), and in high density Cylinders 3 (C) and 4 (D).

The nitrite concentration in low density replicate cylinders showed the maximum concentration at the 20th day of experiment (0.035 mg/L) in Cylinder 1 and at the 18th day of experiment in Cylinder 2 (0.036 mg/L). High density replicate cylinders exhibited higher concentrations (0.036 at the 17th and the 20th day in Cylinder 3; 0.038 at the 17th day in the Cylinder 4).

Phosphate concentrations in low density replicate cylinders reached a maximum value of 0.32 mg/L in Cylinder 1 and 0.29 mg/L in Cylinder 2 at the 21st day of experiment. Phosphate concentrations in high density replicate cylinders reached a maximum value of 0.33 mg/L in Cylinder 3 and 0.38 mg/L in Cylinder 4 at the 21st day.

Ammonium, nitrite and phosphate concentrations showed linearly increasing trends among low density and high density replicates. Ammonium, nitrite and phosphate concentrations exhibited significant differences among low and high density replicates (ammonium concentration: Wilcoxon matched-pairs test, $p < 0.001$; nitrite concentration: Wilcoxon matched-pairs test, $p < 0.01$; phosphate concentration: Wilcoxon matched-pairs test, $p < 0.01$).

Survival rates showed a linearly decreasing trend in each cylinder, reaching, in both treatments, minimum values at the 16th day (Fig. 2.4). In particular, at the day 16th, the survival in Cylinder 1 was 70.0% (± 8.7) of the initial larval density; the survival in Cylinder 2 was 65.0% (± 5.0) of the initial larval density; the survival in Cylinder 3 was 46.7% (± 12.0) of the initial larval density; the survival in Cylinder 4 was 57.8% (± 1.9) of the initial larval density. Larval survivorships of each replicate after the 16th day of experiment could not be evaluated because at the end of the larval development, last-stage zoeae started the settlement and they were no more sampled in the water column.

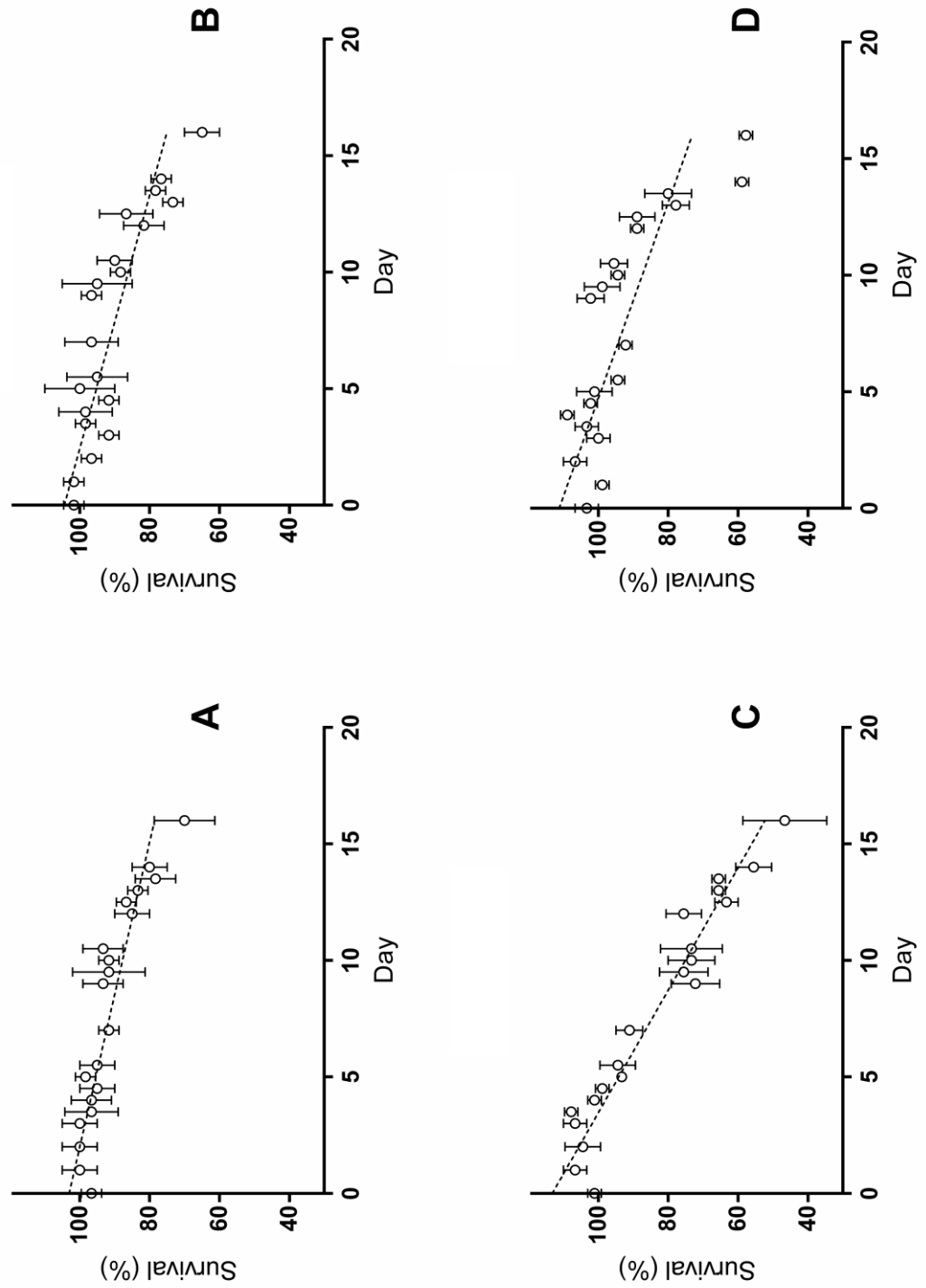


Fig. 2.4: Percent survival rates of *Hippolyte inermis* larvae cultured 21 days in the automatic system in each culture cylinder (dots) and their standard deviations (vertical bars).

At the end of the first experiment (day 21st) the specimens still present in each replicate cylinder were collected (Fig. 2.5). In total 318 post-larvae were collected from Cylinder 1 (survival 85.9%; post-larval density 0.086 PL.mL⁻¹); 310 post-larvae were collected from Cylinder 2 (survival 83.8%; post-larval density 0.083 PL.mL⁻¹); 394 post-larvae were collected from Cylinder 3 (survival 71.6%; post-larval density 0.106 PL.mL⁻¹) and 411 post-larvae were collected from Cylinder 4 (survival 74.7%; post-larval density was 0.111 PL.mL⁻¹).

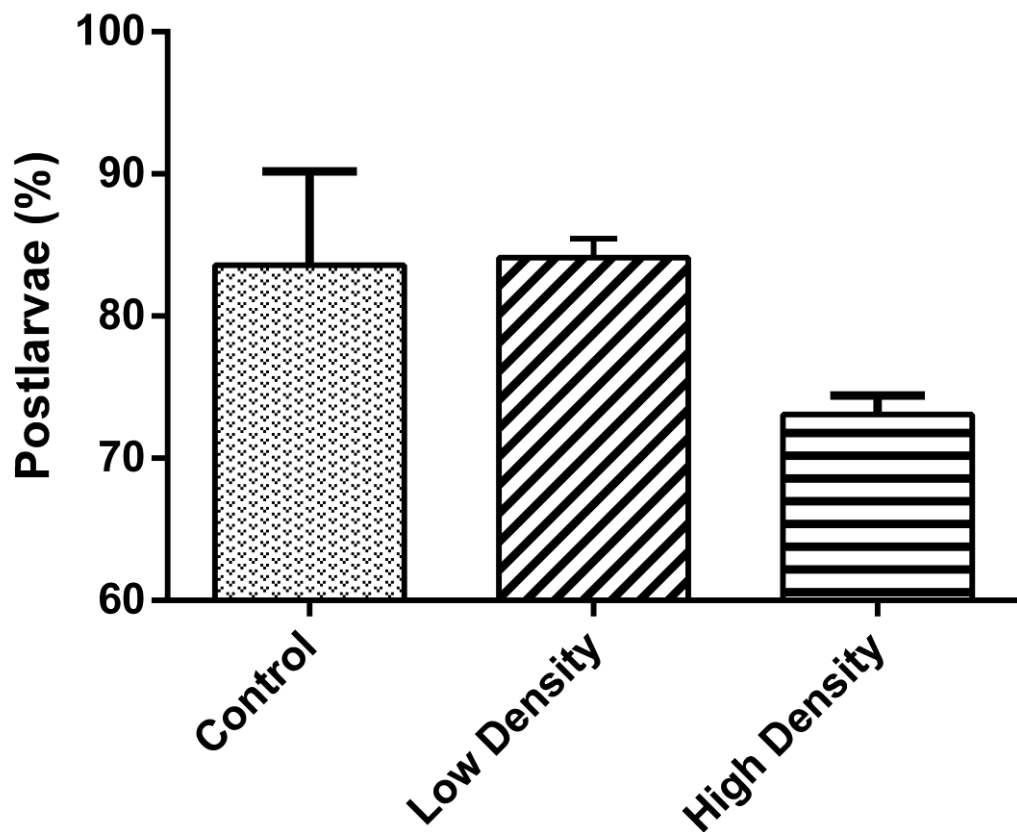


Fig. 2.5: Percentage of post-larvae collected in the control culture replicates, low density replicates (Cylinders 1 and 2) and in the high density replicates (Cylinders 3 and 4); vertical bars indicate standard deviations.

The remaining, non-metamorphosed larvae were collected as well. In total 30 larvae were present in Cylinder 1 (8.1%), 17 larvae in Cylinder 2 (4.6%), 43 larvae in Cylinder 3 (7.8%) and 16 larvae in Cylinder 4 (2.9%). At the end of the second experiment we collected 311 post-larvae from Cylinder 1 (survival 84.1%; post-larval density 0.084 PL.mL⁻¹), 306 post-larvae were collected from Cylinder 2 (survival 82.7%; post-larval density 0.083 PL.mL⁻¹), 404 post-larvae were collected from Cylinder 3 (survival 73.5%; post-larval density 0.109 PL.mL⁻¹) and 399 post-larvae were collected from the Cylinder 4 (survival 72.5%; post-larval density 0.108 PL.mL⁻¹). Non metamorphosed larvae were collected in the cylinders 1, 2, 3, 4 and they were 5.1%, 5.4%, 8%, 5.5% of the initial stocks, respectively. Average survival in replicates conducted at a low density was 84.13% (± 1.33) while in replicates conducted at a high density was 73.08% (± 1.33). No significant differences among low density and control culture replicates in the survival rates were recorded (Mann Whitney test, $p > 0.05$). In contrast, significant differences among low density and high density replicates were recorded in the survival rates (Mann Whitney test, $p < 0.05$).

2.1.4.2 CONTROL LARVAL CULTURE

The control experiment, conducted without the aid of an automated device, according to the standard operation procedures proposed by Zupo (2000), consisted of sixteen replicates of 800 mL conical flasks at a density of 0.1 larvae.mL⁻¹, and it yielded high survival values. The survival curve showed a sigmoidal shape. Its slope was deepest from day 3 to day 10 and then it became shallower until the end of the experiment. Considerable differences in survival rates among the replicates can be inferred by the high standard deviation in the survival curve from day 4 to the end of the experiment. At the end of the experiment (day 21st), the average survival obtained in the control experiment was 83.6% (± 6.6) with a density of 0,091 postlarvae.mL⁻¹ (Fig. 2.6). The

highest survival rates were observed in the conical flask 5 (93.8%) while the lowest survival rates were obtained in the conical flask 10 (70%).

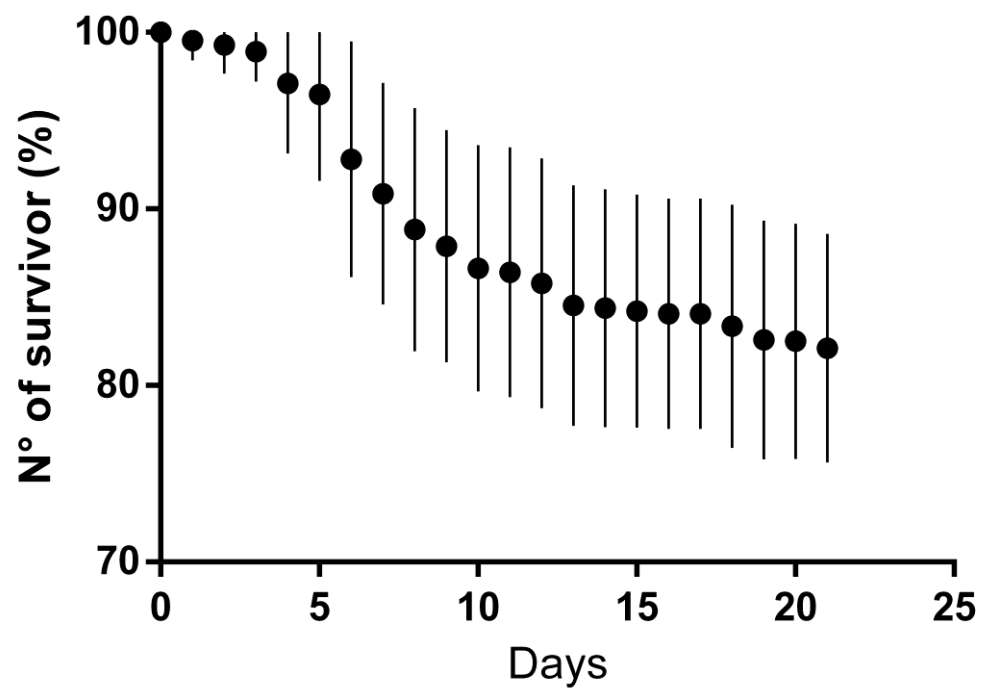


Fig. 2.6: Percent survival rates of *Hippolyte inermis* larvae cultured 21 days in glass vessels managed by operators (dots) and their standard deviations (vertical bars).

2.1.5 DISCUSSION

Larval culture of *Hippolyte inermis* is a strenuous activity. The survival rates recorded are in line with previous studies (Zupo, 2000), although large differences were observed among replicates. The differences in survival among replicates might be due to the difficult manipulation of fragile zoeae (Luis-Villaseñor et al., 2012), the decay of water quality (Robertson and Austin, 1998) or bacterial diseases influencing the mortality in individual replicates (Robertson and Austin, 1998, Luis-Villaseñor et al., 2012). The non-automatized procedures, still assuring a high post-larval production (Zupo, 2000), exhibit some critical issues: a thermostatic chamber is needed, as well as plenty of space and trained operators (Calado et al., 2008). For this reason, an automatic larval system has been set to work without the intervention of trained biologists and it does not need an intensive daily maintenance. According to Calado (Calado et al., 2003, Calado et al., 2005, Calado et al., 2008) the automatic culture system has been devised in order to meet the needs of cultured species and reduce research and laboratory resources while optimizing spaces and the use of manpower.

During water changes, both water inflow and outflow were activated simultaneously, so avoiding changes in the water level that could lead to mortality (personal observation). In addition, larvae were not forced to pass through or pressed on the meshes, due to the pressure of the outflowing water (Quinitio et al., 1999, Nghia et al., 2007).

As for the chemical properties of the water, temperature and pH are the most important parameters influencing larval survival. Stable temperatures and pH were recorded during the experiment. These parameters can modify larval physiology with consequence on health, residual energy, difficulties in molt and settlement (Palma et al., 2009, Taylor et al., 2015). The concentrations of waste compounds were constant during the experiment

and, consequently, water analyses in tank B (sterilization tank) yielded constant concentrations, also due to the intensive ozonisation and filtration provided.

In spite of inorganic pollutants recorded in tanks A and B, tanks C1 and C2 (culture tanks) were influenced by the presence of larval culture. Tanks C1 and C2, in fact, exhibited very significant differences in the daily concentrations of ammonia. These differences may be correlated to the presence of high density cultures in the tank C2.

In addition, decomposing brine shrimps may induce higher concentrations of POM and DOM and, consequently, of nitrogen compounds deriving from their degradation. The accumulation of organic material can lead to a saprophytic, potentially pathogenic, bacterial proliferation and to a consequent decrease of dissolved oxygen (Leonard et al., 2000). In the equipment we tested, bacteria were controlled by the activity of protein skimmers (Brambilla et al., 2008; Suzuki et al., 2008), as well as the disinfection of incoming water (in tank B) and this reduces the bacterial growth in the filter units.

Ozone addition is effective against a wide range of bacterial, viral, fungal and protozoan pathogens. The effectiveness is concentration-dependent and is influenced by the exposure time, pathogen loads and abundance of organic matter (Gonçalves and Gagnon, 2011). Although seawater ozonisation may generate bromate ions as a by-product (Parrino et al., 2015), during our experiment we did not find any evidences of larval mortality induced by the disinfection process.

Density of food and larvae is a key point for the success of any culture. Larvae must be fed *ad libitum* and, usually, organisms are cultured in a medium containing high food concentration, creating potential problems due to decaying organic matter and possible decrease of dissolved oxygen concentrations. High density replicates showed significantly higher concentrations of nitrogen compounds and phosphates as compared to low

density replicates. The maximum concentration of nitrite and phosphate ions in high density replicates was slightly higher than in the one of low density replicates. As well, the maximum concentration of ammonia was considerably higher in high density replicates than low density replicates.

These differences, which increased during the latest days, can be explained by considering that in the semi-closed system, larval units exchanged water only for a few hours each day. During this time the aged, dying or dead feeds were removed. The ammonium concentration was, at least partially, related to the larval density, since the concentration of live feeds is identical in the two treatments.

Nitrogen compounds did not reach lethal concentrations: LC50 (96 h) analysis on decapod crustacean larvae and juveniles showed a species-specific toxicity of nitrogen compounds (Liao et al., 2011, Romano and Zeng, 2013). These studies were performed on species that are phylogenetically far from *H. inermis*. LC50 concentrations measured in above-mentioned studies were generally higher than those recorded in our treatments. However, other processes could influence the mortality in the described equipment, in the case of organisms with a longer period of larval development (Schuenhoff et al., 2003).

The larval survival rates in cylinders showed a linear trend from the day 0 to the day 16; after this time, the number of sampled larvae decreased, due to both behavioural and morphological changes (Zupo and Buttino, 2001). In fact, latter zoeal stages are characterized by the elongation of pleopods and pereopods, positive phototaxis and by the tendency to settle to the substrate, as a transition period preceding the benthonic phase, thus decreasing the probability to collect swimming larvae into the culture units. For these reasons, it was impossible to obtain a complete estimation of the larval density from the day 17th to the end of the experiment in the automated culture equipment. Low

density replicates and control culture replicates showed the same post-larval production trends and the differences among them are not significant. In general, the survival rates in the automatic equipment were quite high, with higher survivals in low density replicates, due probably to lower concentration of pollutants, higher food availability and reduced influence of cannibalism. Despite the statistical differences recorded for the survival rates among low density and high density replicates, these last showed lower survival rates but they produced a higher number of healthy larvae per volume unit.

The efficiency of the described automatic culture equipment depends, as demonstrated in other rearing systems (Calado et al., 2003, 2005, 2008), on various chemical, biological and mechanical influences. The efficiency was improved making use of a filter, frequent water changes, a correct dosage of feeds, the use of ozone and a careful setting of optimal densities. The process was also improved by a fine tuning of the operational software, providing efficient water changes. These improvements conducted to results similar to those produced by manually operated culture system, with lower operational efforts and costs. This automatic equipment largely simplifies the culture of small laboratory organisms and it offers interesting applications for the purposes of aquaculture and scientific or biotechnological research (Nappo et al., 2012).

3 : ECO-TOXICOLOGICAL TESTS

3.1 SPECIFIC TOXICITY OF *HALOMICRONEMA METAZOICUM* (CYANOPROKARYOTA, CYANOPHYTA)

EXUDATES

3.1.1 ABSTRACT

Cyanobacteria are a phylum of prokaryotes widely distributed in aquatic and humid environments. They may live, as individuals or colonies, in the water column and in benthos as well as they can establish symbiotic relationship with other organisms, and are known to produce a wide spectrum of bioactive substances. In particular, the ecological implications of *Phormidium*-like Cyanobacteria are still underexplored, but these organisms are known to produce a variety of biological active molecules influencing and interacting with plant and animal organisms living in their own environment, both in freshwater and marine ecosystems. We have isolated a free-living strain of this *Phormidium*-like Cyanobacterium from the leaves of the seagrass *Posidonia oceanica* (L.) Delile. Morphological investigations indicated it is *Halomicronema metazoicum*, a species previously known as a symbiont of the marine spongiae *Petrosia ficiformis*. Interestingly, preliminary observations indicated that the exudates of this cyanobacteria culture are extremely toxic for various protozoans and fish parasites, but non-lethal for fish, at the same doses. We still ignore if cyanobacterial mats contain constitutive or activated defences influencing the physiology of other organisms, besides the observed activity of their spent medium.

For this reason, we cultured the strain at 22 °C, with a photoperiod 12/12h and light irradiance 140 µE. The bioactivities of its spent culture medium and of Cyanobacteria mats were measured using standard toxicity tests on *Artemia salina* nauplii to investigate

if the active compound(s) are present a) in the benthic mat tissues and available after wounding and/or b) in the culture medium, while produced as exudates.

In addition, we cultivated our cyanobacteria in a range of light irradiances, temperatures and salinities, to establish the most suitable conditions for the production of allelopathic and toxic compounds. The bioactivity of its spent culture medium was measured by performing standard toxicity tests on two model organisms.

Our results indicate that wounded cyanobacteria masses, once crushed, do not contain any toxic activity. In contrast, a high toxicity of their spent culture medium was detected and the activity was determined at a range of concentrations. We observed that at least two bioactive compounds are produced, at low and high irradiance levels and at two temperatures. The main compounds influencing the survival of model organisms are produced at the highest temperature and high or intermediate irradiance levels.

Thus, these organisms commonly found both in planktonic communities and on benthic substrates (either as free-living organisms or endosymbionts of invertebrates) may play an important ecological role, possibly influencing the ecology of complex environments as *P. oceanica*, ruling the survival and the seasonal blooms of various species through the production of constitutive toxic exudates. The interesting ecological relationships we are just discovering and the demonstrated specific biological effects of *H. metazoicum* represent important steps towards the development of new biotechnologies in the field of aquaculture, ecological conservation and medicine. Future isolation, identification and production of bioactive compounds will permit exploitations for biotechnologies in the fields of ecological conservation and medical applications.

3.1.2 INTRODUCTION

Cyanobacteria, or blue-green algae, are prokaryote photosynthetic bacteria distributed in a large variety of environments (Dahms et al., 2006), from freshwater to marine and terrestrial ecosystems (Gaylarde et al., 2004). These prokaryotes, living either as unicellular or colonial forms, are characterized by remarkable taxonomic diversity (Usher et al., 2004; Whitton, 1992) and an even more notable functional diversity (Barberousse et al., 2006). They are classified with algae due to their chlorophyll a and other compounds content, and as bacteria, due to several structural features resemble to those of bacteria (Khatoon et al., 2018). They are among the earliest known form of life on Earth and were the first photosynthetic organisms, responsible of the production of oxygen in the early atmosphere (Canfield, 2005; Berman-Frank et al., 2003; Kasting and Siefert, 2002). Cyanobacteria are among the most studied organisms in the drug mining field due to the potential as producers of bioactive molecules such as chlorophyll a, b and c, carotenoids and phycobilins as well as source of antioxidant compounds (Begum et al., 2016; Patel et al., 2006) and, in certain culture conditions, some species are able to produce bioplastic poly-3-hydroxybutyrate (Taepucharoen et al., 2017). They are highly fluorescent, due to the presence of phycobiliproteins, proteins with covalently attached linear tetrapyrrole pigments (Paliwal et al., 2017). Different types of cyanobacteria have been recognized as a source of a large number of bioactive molecules (Dahms et al., 2006; Abarzua et al., 1999) and most of them consist of amino acids and fatty acids often with anti-bacterial, anti-fungal, anti-algal or anti-protozoan activities (Khatoon et al., 2018; Kurmayer et al., 2016; Cheung et al., 2013; Nagarajan et al., 2012; Dobretsov et al., 2011; Dahms et al., 2006). In addition, physiologic differences among strains may largely encompass the dissimilarities among species and genera (Pfeiffer and Palińska, 2002) and their noteworthy diversity also explains the importance as potential producers of novel

bioactive substances with economic potential (Blunt et al., 2003; Shimizu, 2003). Among Cyanobacteria, species belonging to the *Phormidium* group are a source of a large variety of compounds such as lipids and polysaccharides, sterols, proteins, vitamins and enzymes (Derby and Sorensen, 2008; Pulz and Gross, 2004). From an ecological point of view, cyanobacteria are able to colonize a wide range of habitats thanks to their toxigenic and teratogenic capability against a vast range of grazers or competitors (Dias et al., 2017; Manivasagan et al., 2017; Borges et al., 2015), or due to their ability to affect and modify chemical characteristics of water (Jüttner et al., 2001). Despite the huge quantity of chemicals produced by cyanobacteria, they can be also associated in symbioses with animals and algae such as the common association with demospongiae, corals and other invertebrates in marine environment (Zehr et al., 2016; Caroppo et al., 2012). In the *Phormidium* group, species of the *Halomicronema* genus are well-known symbionts of other organisms, such as the cave coral *Oculina patagonica* (Koren and Rosenberg, 2008), the massive coral *Goniastrea aspera* (Rosenberg and Ben-Haim, 2002) and the stony sponge *Petrosia ficiformis* (Konstantinou et al., 2018; Caroppo et al., 2012). Recent observations demonstrate that *Halomicronema metazoicum*, a filamentous non-heterocystous cyanobacteria symbiont of *P. ficiformis* (Caroppo et al., 2012), can live as free-living organism associated to leaves of the seagrass *Posidonia oceanica* (Ruocco et al., in press). *Phormidium* cyanobacteria are well-known to produce a wide range of toxins (Dias et al., 2017; Wood et al., 2017), such as anatoxins, microcystins, saxitoxins and portoamides (Borges et al., 2015; Kouzminov et al., 2007; Gugger et al., 2005; Teneva et al., 2005), and these compounds are produced by cyanobacteria to face the complexity of interactions among communities associated to seagrass leaves (Mazzella et al., 1992; Devlin et al., 1977).

In this work we investigated the bioactivity of *Halomicronema metazoicum* to check if active compound(s) are present a) in the benthic mat tissues and available after wounding and/or b) in the culture medium, while produced as exudates, and, in addition, if the toxic compounds, present in the culture medium, is stable in the water for 24 hours. The investigation were conducted through a bio-assay on standard model organisms, *Artemia salina*, using standard toxicity tests (Lopes et al., 2010; Beattie et al., 2003; Parra et al., 2001; Kiviranta et al., 1991). Since the relevance of culturing cyanobacteria also derives from their ability to produce compounds for biotechnological applications (Gerçe et al., 2009; Moore et al., 1988), and the production of toxic compounds is often modulated by salinity, light irradiance and temperature (Caroppo et al., 2012), the aim of this study is, also, to characterize the environmental conditions maximizing the production of allochemicals and toxins produced by *Phormidium*-like cyanobacteria (Dias et al., 2017).

3.1.3 MATERIALS AND METHODS

3.1.3.1 COLLECTION OF CYANOBACTERIA SAMPLES AND MEDIUM PRODUCTION

Cyanobacteria mattes were collected from *P. oceanica* leaves in the seagrass meadow off Lacco Ameno d'Ischia (Bay of Naples, Italy. 40°44'56" N, 13°53'13" E), and were transferred to sterile multi-well dishes filled with 4 mL of f/2 medium (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). The cultures were re-inoculated and checked for the presence of foreign or contaminating species various times up to a complete purification. The axenic condition of the culture was determined by means of SEM and light microscopy. Cyanobacteria were identified as *Halomicronema metazoicum* by means of morphological analyses, in collaboration with Professor Antonino Pollio of the University of Naples Federico II, and molecular tools, in collaboration with Dr. Maria Costantini of the Stazione Zoologica Anton Dohrn. Axenic cultures were maintained at 22 °C with an irradiance of about 200 μ E and a photoperiod (dark/light) of 12/12 hours in f/2 medium. Cultures were re-inoculated every 20 days to avoid evaporation, nutrient deficiency and accumulation of pollutants.

Five small fragments of 5.0 (\pm 0.3) grams of fresh weight of *Halomicronema metazoicum* were isolated from mother cultures and individually cultivated in sterile 2 L Erlenmeyer flasks containing 1.5 L of f/2 medium at the same conditions described for mother cultures. Culture media and small fragments have been collected at the end of the cultivation period (40 days), the media filtered using a Stericup Filter Units (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), fragments were collected, grinded, potterized, each homogenate diluted in 50 mL of filtered and sterilized seawater. In the 40 days of culture, the fresh weights of each fragment were estimated.

To optimize the production of bioactive molecules, small portions of cyanobacteria mattes were collected from mother cultures, cut in pieces of about 5 g (fresh weight) and

individually cultured in 2 L Erlenmeyer flasks containing 1.5 L of *f/2* medium. Two replicate flasks were cultivated under each condition of light, temperature and salinity, according to a factorial experimental plane (Table 3.1) containing 27 combinations.

Treatment Condition	Salinity ‰	Irradiance μE	Temperature °C
1	36	80	16
2	40	80	16
3	44	80	16
4	36	140	16
5	40	140	16
6	44	140	16
7	36	200	16
8	40	200	16
9	44	200	16
10	36	80	18
11	40	80	18
12	44	80	18
13	36	140	18
14	40	140	18
15	44	140	18
16	36	200	18
17	40	200	18
18	44	200	18
19	36	80	22
20	40	80	22
21	44	80	22
22	36	140	22
23	40	140	22
24	44	140	22
25	36	200	22
26	40	200	22
27	44	200	22

Table 3.1: Experimental conditions imposed to the culture of cyanobacteria to obtain three combinations of salinity (36, 40, 44), irradiance (80, 140, 200 μE) and temperature (16, 18, 22 °C) for the toxicity test treatments.

In particular, we tested values of salinity, temperature and irradiance that could be found in various habitats of the Mediterranean Sea and factorially combined them to test the production of bioactive compounds. Cyanobacteria were cultivated 40 days in these conditions, to facilitate an accumulation of secondary metabolites in the spent medium. Culture media were collected at the end of the production period, filtered over a 0.22 μm Millipore filter, stored in glass vessels and kept at -20 °C up to the start of bioassays.

3.1.3.2 TOXICITY TESTS ON *ARTEMIA SALINA*

Toxicity tests were conducted on nauplii of *Artemia salina*, a standard model organism used for a wide range of eco-toxicological inquiries (Garaventa et al., 2010; Nunes et al., 2006; Persoone and Wells, 1987). Cyanobacteria spent culture media and mattes homogenate were pooled and diluted at various concentrations in filtered seawater to prepare five dilutions of the two treatments: 1:5, 1:10, 1:100, 1:1000, 1:10000, in volume.

In addition, we prepared replicates at the same five dilutions of spent culture media but exposing the test organism *Artemia salina* for 5 minutes to check the activity of cyanobacteria compounds, among time. 5-minutes threatened nauplii were then transferred to multi-wells filled with sterile seawater up to the end of the experiment.

Commercially available dehydrated cysts of *Artemia salina* were used for the experiments (Super high group SRL, Ovada, Alessandria, Italy). To obtain nauplii, 500 mg of cysts were incubated for 24 h at 27°C under 12 h light, 12 h dark conditions and continuous aeration of the cyst suspension in seawater (38‰ salinity). Newly hatched nauplii were isolated from non-hatched cysts thanks to their positive photo-taxis and then collected and transferred into a beaker, filled of fresh seawater, in a final concentration of 20 larvae/mL. Twenty nauplii were transferred into 4 replicates of 5 mL multi-well plates filled with 4 mL of solution (spent medium diluted in seawater) for each of the above-mentioned

concentrations and treatments, and their motility and survival rates were checked at 5 min, 60 min, 300 min and 24 h. Negative controls were prepared adding fresh *f/2* medium.

3.1.3.3 OPTIMIZATION OF BIOACTIVE MOLECULES PRODUCTION

To obtain the best culture condition optimizing the production of bioactive molecules, toxicity tests were performed on two model organisms: adults of the rotifer *Brachionus plicatilis* (Snell and Carmona, 1995) and embryos of the sea urchin *Paracentrotus lividus* (Romano, 2003). The contents of two replicate samples produced for each treatment were pooled prior to the tests. The spent medium was sampled and diluted at various concentrations in filtered seawater, according to the tested models. Basically, we took into account the following six dilutions of the culture medium in filtered seawater: 1:5, 1:10, 1:100, 1:1000, 1:10,000, 1:100,000 in volume. However, in the case of *P. lividus*, the highest concentrations (1:5, 1:10, 1:100) were not considered in further analyses, because they produced immediate mortality of sensible embryos (Romano, 2003). In the case of *B. plicatilis* the lowest concentrations (1:1000, 1:10,000, 1:100,000) were not considered in further analyses, because they did not produce any effect on this organism, able to partially detoxify poisons (Dahms et al., 2011).

To perform tests on rotifers, a single clone of *B. plicatilis* maintained in continuous culture at the Stazione Zoologica Anton Dohrn has been used. This clone continuously produces offspring when cultivated at 20 °C and it is fed on cultures of *Dunaliella* sp. replaced every 5 days. As above referred, three concentrations of spent medium were considered for bioassays on *B. plicatilis*, i.e., 1:5, 1:10 and 1:100 in volume, after preliminary tests indicating concentrations lower than 1:100 did not produce any effect in respect to controls (Dahms et al., 2011). Fifty adult individuals of *B. plicatilis* were transferred into 3 replicates of 5 mL multi-well plates filled with 4 mL of solution (spent medium diluted in

seawater) for each of the above-mentioned concentrations, and their motility and survival rates were checked at 5 min, 60 min, and 24 h, for each of the experimental conditions.

Three dilutions of spent medium were considered for bioassays on *P. lividus* embryos, 1:1,000, 1:10,000 and 1:100,000, in volume. Higher concentrations (1:5, 1:10, 1:100) produced immediate block of embryo development at the first division and they were not further considered in our analyses. The test was prepared starting from two mature females and one male of *P. lividus* collected in the Bay of Napoli. Sea urchins were injected 1 mL of 0.5M KCl into the coelom through the soft derma around the mouthparts, to stimulate the contraction of gonads. They were vigorously shaken and females were placed with their mouths up, over a 50 mL beaker until the gametes were released into filtered (0.22 μ m Millipore) seawater, to facilitate the collection of eggs, that were rinsed three times with clean seawater to remove possible organic residuals (Chapman, 1995). Sperms were collected “dry”, using a Pasteur pipette and sucking over the surface of male gonopores, to avoid premature activation. The gametes obtained from each individual were conserved in plastic vessels until fertilization. Sub-samples of eggs were collected and added with a drop of sperm suspension. Egg activation was revealed by the elevation of the fertilization membrane within 40-80 s, appearing as a clear circle. Pools of embryos exhibiting percentages of fertilization lower than 95% were discarded. Pools exhibiting viable embryos were used for bioassays. To this end, groups of 500 embryos obtained from each replicate female were collected in duplicate and transferred into 5 mL multi-well dishes filled with appropriate dilutions of the cyanobacteria culture media, as above specified.

Four replicate tests were run at each concentration of the spent culture medium, plus four negative controls prepared using only seawater added with corresponding

proportions of fresh $f/2$ medium. The results were recorded at various time intervals and according to each concentration. In particular, the multi-wells were observed after 1 hour under the inverted microscope to record the percentage of individuals showing normal cell division and, eventually, the presence of apoptosis hallmarks, such as blebbings. After 6 h and 24 h, 1 mL of egg suspension was fixed with the addition of a drop of 40% buffered formalin and replicates examined to record the percentage of individuals at the blastula, gastrula and prism stages, respectively. The percentage of individuals that were still at the first stages of divisions and those blocked or in apoptosis was recorded as well. The remaining content of wells was fixed after 48 h and examined to record the percentage of normal plutei.

3.1.3.4 STATISTICAL ANALYSES

Data were organized as means with standard deviations from various replicates, for each set of measurements.

As regard *Artemia* toxicity tests, raw data were analysed using two-way ANOVA and Dunnett's multiple comparisons post-hoc tests were applied to check the differences against negative controls of each treatment at each time. Spent medium and 5-minutes threated replicates were analysed using two-way ANOVA, checking the differences between replicates at the same dilution and at the same experimental time. Data were tested for normality by the Shapiro-Wilk normality test.

As regard the optimization of the bioactive molecule productions, Raw data were analysed using two-way ANOVA and Tukey's multiple comparisons post-hoc tests were applied to check the contribution of each factor to the differences observed. Newman-Keuls test was performed in addition to above-mentioned analyses to detect differences

among treatment means. Student's *t*-test was used to check the significance of differences between individual treatments.

Data were tested for normality and homogeneity of variances by the Kolmogorov-Smirnov and Levene's tests, respectively. Graphs and statistical analyses were computed using GraphPad Prism version 7.00 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

3.1.4 RESULTS

3.1.4.1 TOXICITY TESTS ON *ARTEMIA SALINA*

Cultures of *Halomicronema metazoicum* isolated and cultivated in this study appeared clean of contaminants or other undesirable organisms. The fragments produced in the first 20 days an average biomass of 7.37 grams (± 0.6) with a daily increment of fresh weight of 2.3% (± 0.3) and a final average biomass of 10.7 grams (± 1.3) with a daily increment of 3.3 % (± 1.0) in the whole culture period.

Treatments administrated with spent culture media and cyanobacteria homogenates produced a different pattern of answers in *Artemia salina* nauplii. A constant number of individuals was recorded in negative controls during the experiment with a survival rates of 98.8% (± 2.5) individuals at 5 minutes, 96.3% (± 4.8) at 60 minutes, 95% (± 7.1) at 300 minutes and 92.5% (± 8.7) at 24 hours.

High survival rate was recorded in homogenate treatments, with the lowest survival obtained in replicates 1:5 at time 24 hours and 83.7% (± 13.8) and 85% (± 10) in replicates 1:10000 at time 300 minutes and 24 hours (Fig. 3.1). Overall, increasing time of exposure produced a slightly increase in mortality (ANOVA, $p < 0.0001$), different dilutions did not produce any evident toxic effect on the *Artemia salina* survival (two-ways ANOVA, $p > 0.05$) and no differences were found between each treatment and negative controls (Dunnett's test, $p > 0.05$) at each experimental time interval.

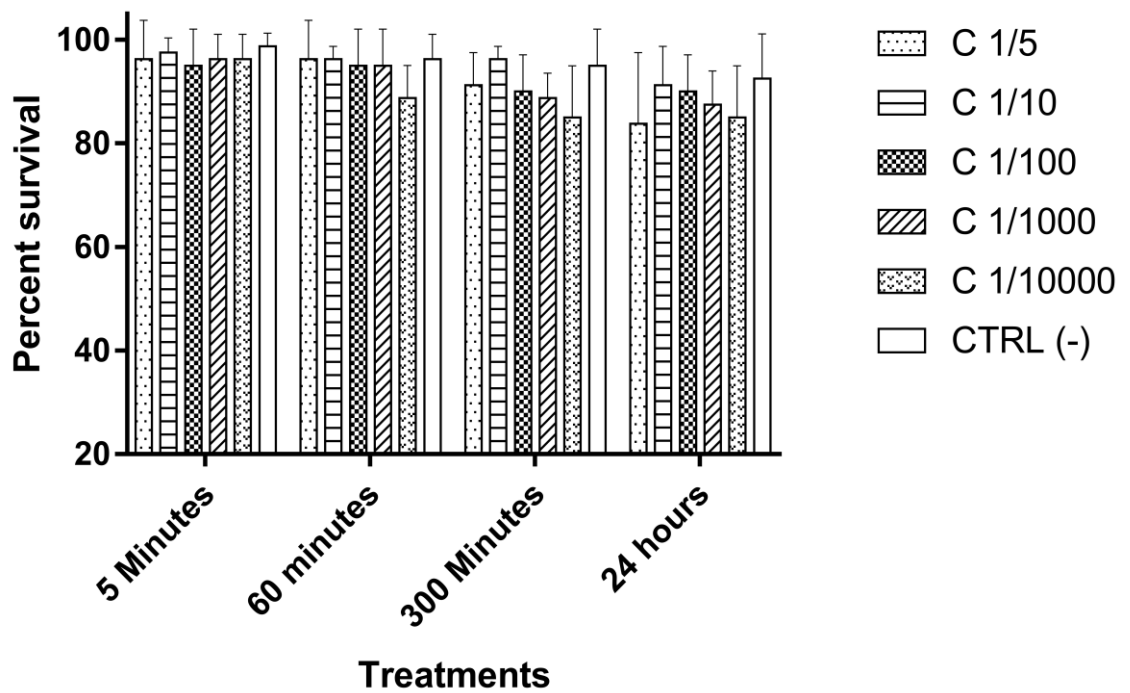


Fig. 3.1: Survival rates of *Artemia salina nauplii* recorded after 5, 60, 300 minutes and after 24 hours of exposure to five concentrations of homogenate of *Halomicronema metazoicum*.

In spent medium treatments (Fig. 3.2), a strong effect of time and dilutions was observed (two-ways ANOVA, $p < 0.0001$). An increasing in survival correlated with dilution was recorded with minimum survival at 1:5 dilution, with a survival rate of 77.5% (± 2.9) at 5 minutes, 60% (± 4.1) at 60 minutes, 41.25% (± 6.3) at 300 minutes and 28.8% (± 8.5) at 24 hours, with significant differences versus negative controls (Dunnett's test, $p < 0.0001$), and maximum survival at 1:10000 with no significant differences versus negative controls (Dunnett's test, $p > 0.05$).

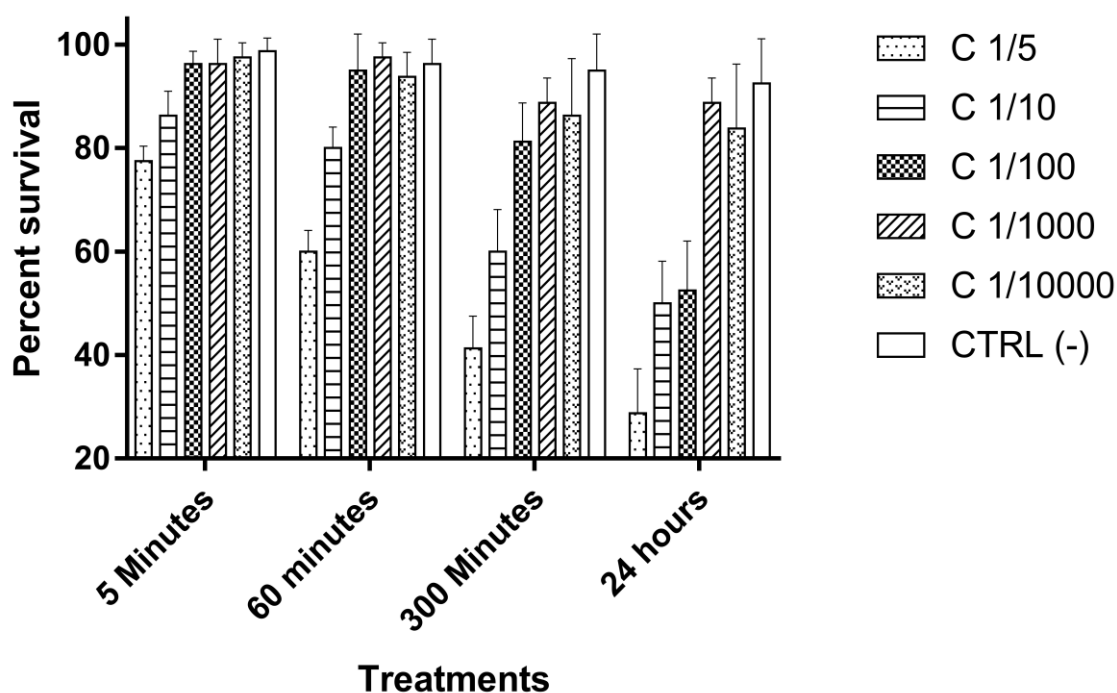


Fig. 3.2: Survival rates of *Artemia salina nauplii* recorded after 5, 60, 300 minutes and after 24 hours of exposure to five concentrations of spent medium of *Halomicronema metazoicum*.

In 5-minutes threated replicates (Fig. 3.3), significant differences were demonstrated in dilutions 1:5 and 1:10 versus negative controls at each experimental time (Dunnett's test, $p < 0.001$), and no significant differences were demonstrated among negative controls and the other dilutions (Dunnett's test, $p > 0.05$).

The comparison among spent culture medium replicates and 5-minutes threated replicates contribute to the understanding of the chemical properties of the bioactive molecules and significant differences among treatments were observed at 300 minutes in dilution 1:5 (Tukey's test, $p < 0.0001$) and 1:10 (Tukey's test, $p < 0.01$) and at 24 hours in dilution 1:5, 1:10 and 1:100 (Tukey's test, $p < 0.0001$).

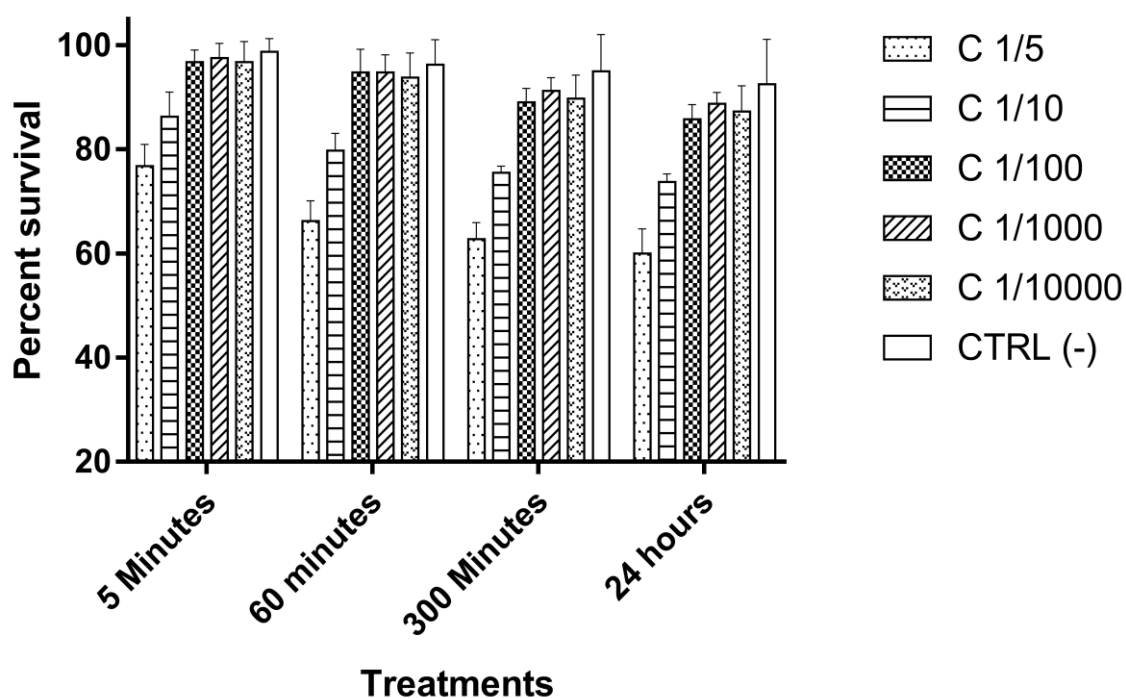


Fig. 3.3: Survival rates of *Artemia salina nauplii* recorded after 5, 60, 300 minutes and after 24 hours in replicates exposed for 5 minutes to *Halomicronema metazoicum* spent medium.

3.1.4.2 OPTIMIZATION OF BIOACTIVE MOLECULES PRODUCTION

The spent culture medium, after 40 days of growth, appeared brownish but transparent. The strains of *Halomicronema metazoicum* isolated and cultured for the purposes of this study appeared clean of contaminants and shaped as dense mattes of non-heterocystous, thin filaments (Fig. 3.4), containing small aggregates of mucous exudates.

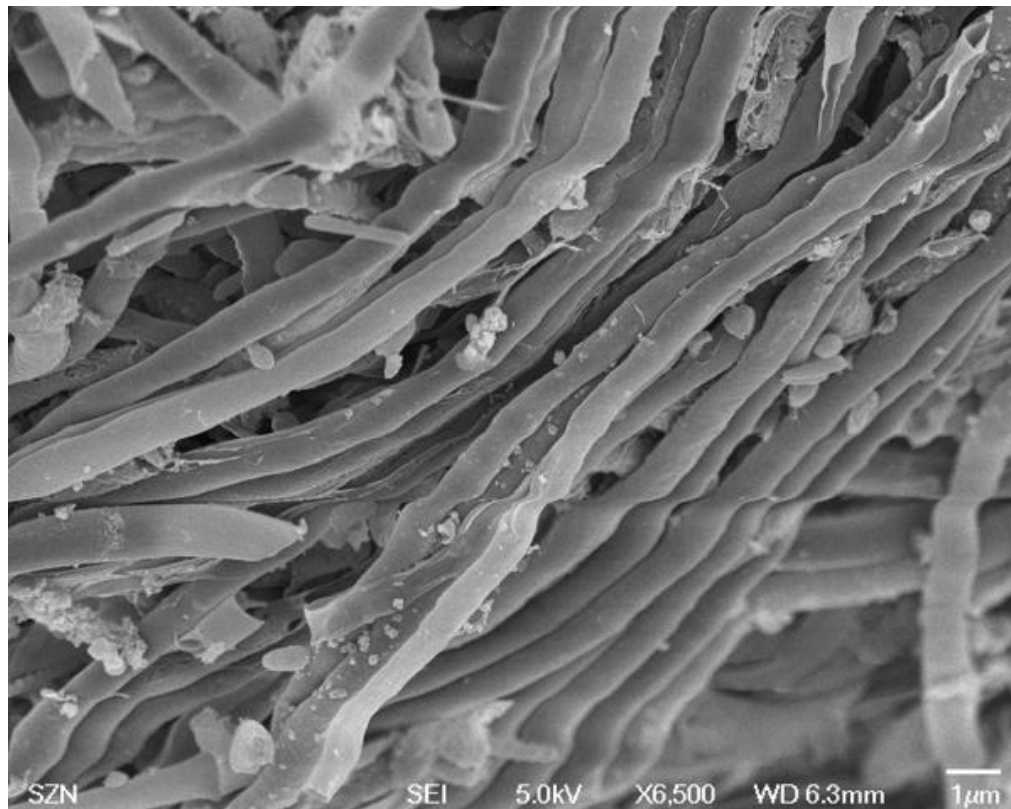


Fig. 3.4 Scanning Electron Microphoto of a sample of cyanobacteria showing a dense mat of non-heterocystous thin filaments. Some small vesicles of amorphous exudates are present on their surface.

Various culture conditions produced complex patterns of answers in *Brachionus plicatilis*, according to time of exposure, temperature, irradiance and salinity. Negative controls (containing fresh *f/2* medium at the corresponding concentrations, as above specified) exhibited an almost constant number of individuals during the experiment and, after 24 hours, the survivorships were still 94% (± 7.21), while the survival rates accounted for 100% at both 5 and 60 minutes after the start of the experiment. Overall, the time of exposure did not produce an evident effect among treatments (ANOVA, $p > 0.05$). A significant difference between the records obtained at 24 h and those obtained at 5 and 60 min (Student-*t*, $p < 0.01$) was demonstrated only in a few conditions (e.g., at salinity 40, irradiance 80 μE and temperature 22 °C). In addition, records obtained at 5 and 60 min

demonstrated no significant differences between them, when analysed by Student-*t* test and in most cases there was no effect on the survival rates compared with those of negative controls (survivals close to 100%). For this reason, it is useful to analyse the results obtained at 24 hours, exhibiting the largest differences (Fig. 3.5).

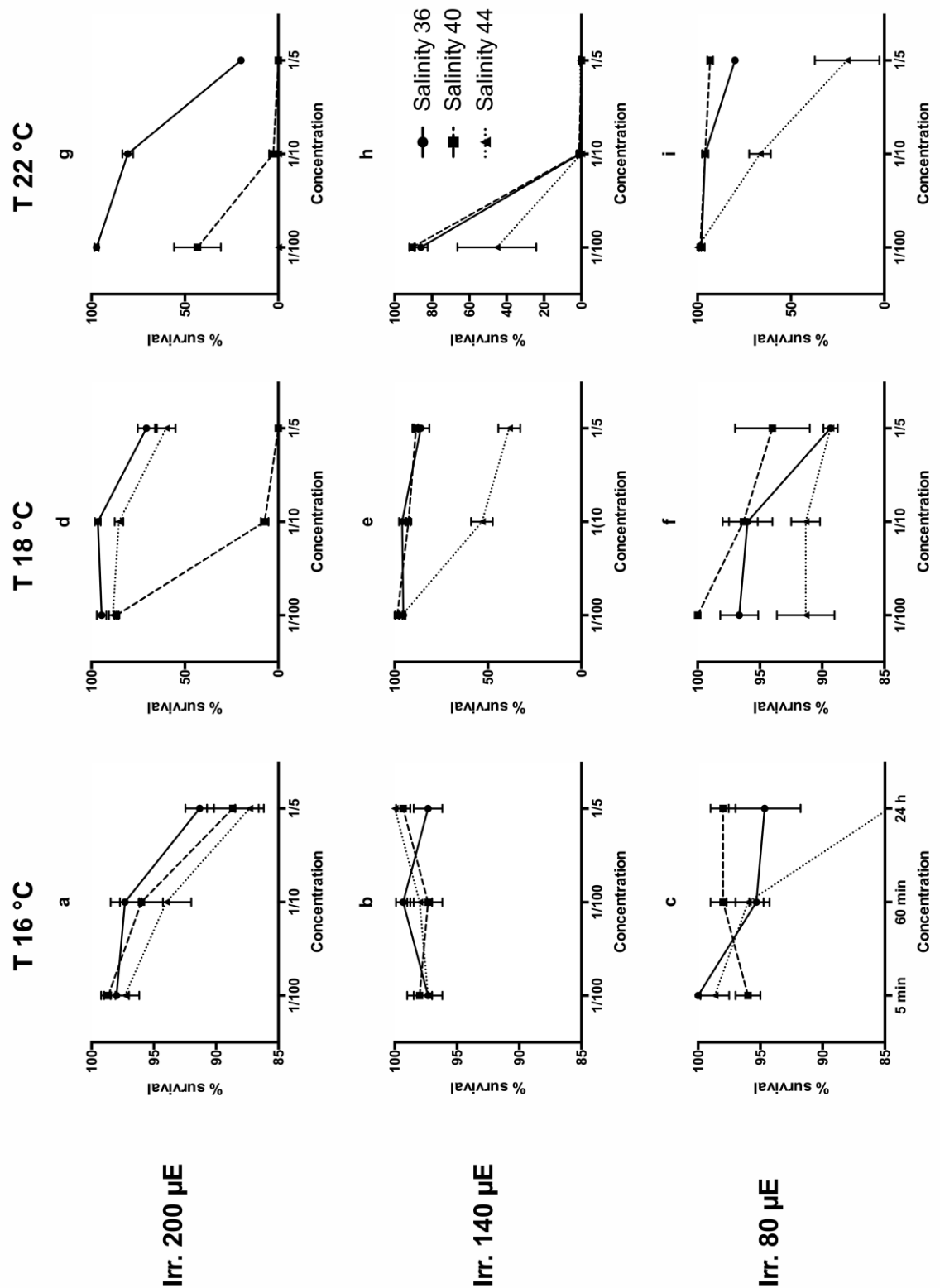


Fig. 3.5: Survival rates of *Brachionus plicatilis* recorded after 24 hours of exposure to three concentrations of the spent culture medium of *Halomicronema metazoicum*, cultivated at three temperatures, three irradiance levels and three salinities.

Survival rates at the highest irradiances decreased in most treatments in a dose-dependent manner, with higher slopes between the concentrations 1:100 – 1:10 and the highest mortalities recorded between 1:10 -1/5 (Fig. 3.5 d, g, h). The factors mainly influencing the differences in mortality rates were temperature and irradiance. Salinity produced significant differences (ANOVA, $p < 0.01$) at 18 °C and 22 °C, especially at the lowest (Fig. 3.5 f, i) and the highest (Fig. 3.5 d, g) irradiance levels. The highest temperatures (Fig. 3.5 g, h, i), salinities and irradiances (Fig. 3.5 d, g) represent the conditions maximizing the production of toxic compounds. Low temperatures (Fig. 3.5 a, b, c) and low irradiances (Fig. 3.5 c, f, i) produced scarce or null toxigenic effects on *B. plicatilis*.

In the case of sea urchin embryos, various development phases offered different results. Negative controls produced 99.0 % (± 0.4) of divided embryos, recorded 1 hour after the *in vitro* fertilization of eggs. In contrast, all treatments, without any significant effect of salinity, temperature or irradiance (ANOVA, $p > 0.05$) demonstrated that the concentration 1/1,000 blocked the development at the first division (Fig. 3.6), while the effect of lower concentration was quite low or null, in all treatments.

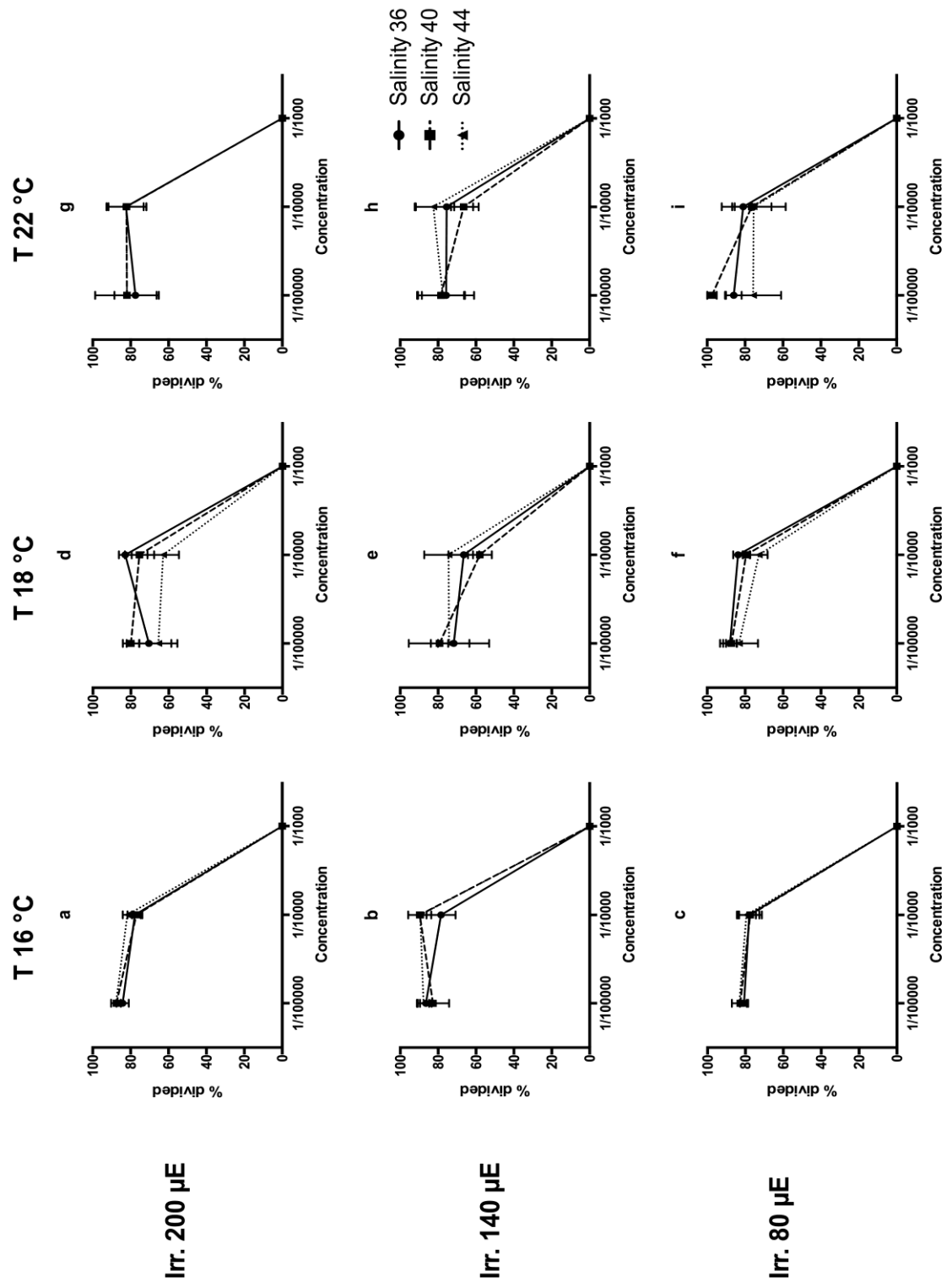


Fig. 3.6: Rates of first divisions of embryos of *Paracentrotus lividus* recorded after 1 hour of exposure to three concentrations of the spent culture medium of *Halomicronema metazoicum*, cultivated at three temperatures, three irradiance levels and three salinities.

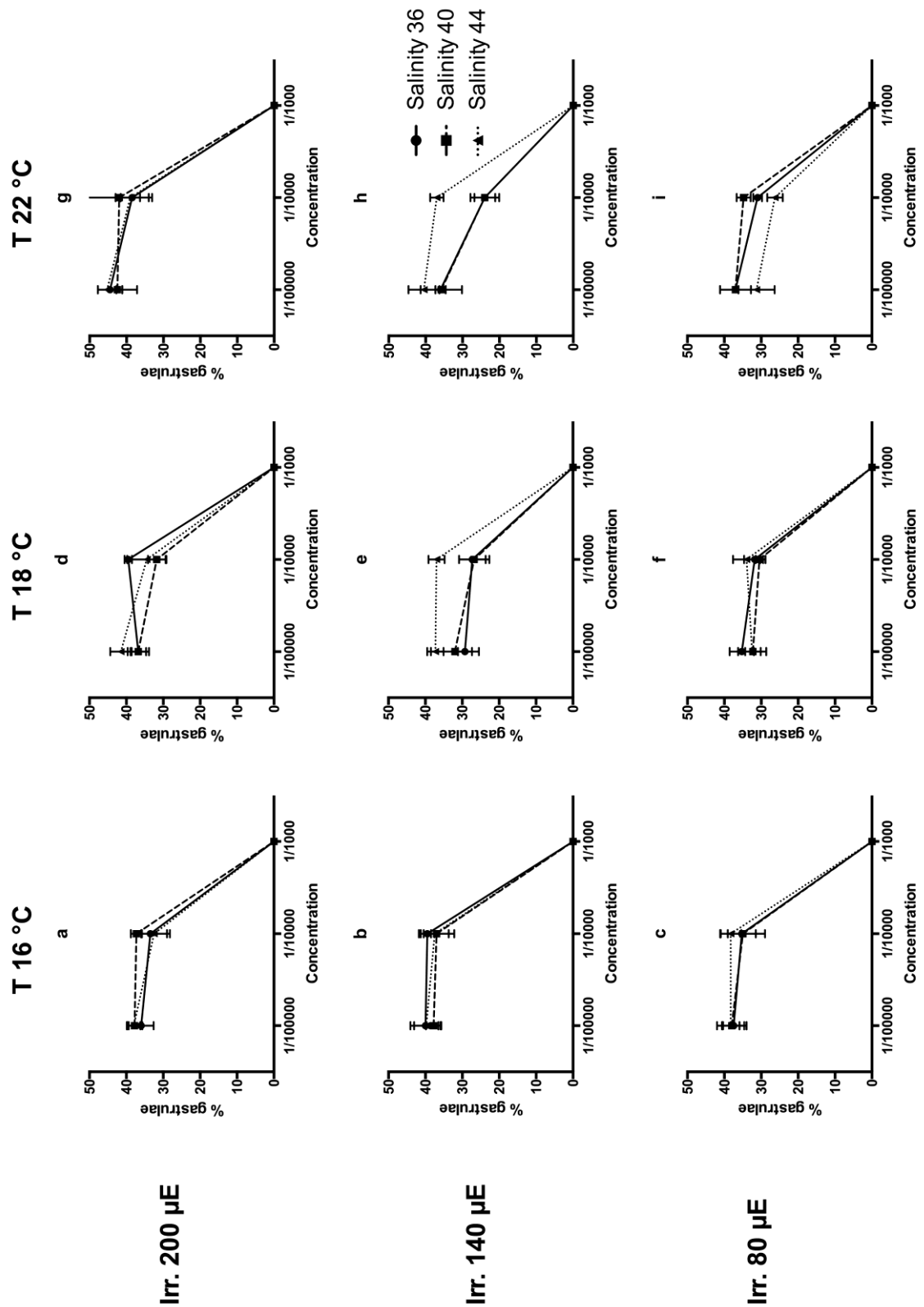


Fig. 3.7: Rates of gastrulation of embryos of *Paracentrotus lividus* recorded after 8 hours of exposure to three concentrations of the spent culture medium of *Halomicronema metazoicum*, cultivated at three temperatures, three irradiance levels and three salinities.

The development of the sea urchin embryos to gastrulae, passing through the stage of blastulae, offered a more complex array of results (Fig. 3.7). However, also in this case a threshold was represented by the concentration 1:1,000, totally blocking or retarding the development, while the concentrations 1: 10,000 and 1:100,000 produced results not significantly different from those exhibited by negative controls. The effect of salinity on gastrulation was generally not significant, with a few differences in various treatments (e.g., Fig. 3.7 e, h, i). As well, the patterns of development in normal plutei were complex, but confirmed the efficacy of the highest concentration (Fig. 3.8).

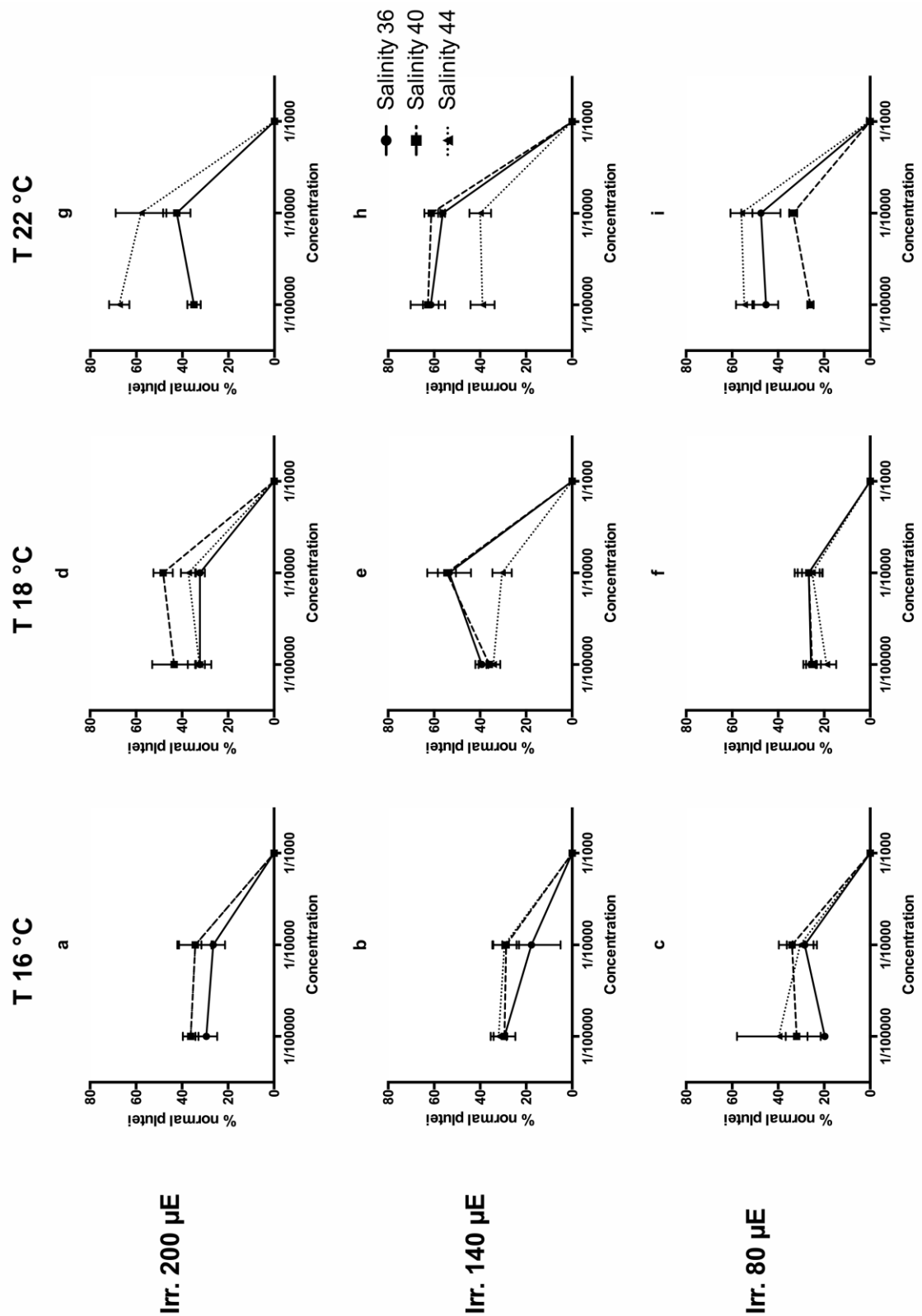


Fig. 3.8: Rates of production of normal plutei of *Paracentrotus lividus* recorded after 48 hours of exposure to three concentrations of the spent culture medium of *Halomicronema metazoicum*, cultivated at three temperatures, three irradiance levels and three salinities.

In this case, the maximum efficacy was recorded at the lowest temperature (16 °C) and at lowest irradiances, generating the lowest percentages of normal plutei (Fig. 3.8 a, b, c, f). Salinity showed contrasting results at the highest temperature. The effective dose was reached consistently between 1:10,000 and 1:1,000.

3.1.5 DISCUSSION

Halomicronema metazoicum is a recently identified species of *Phormidium* group (Caroppo et al., 2012) cyanobacteria symbiont of marine sponge *Petrosia ficiformis* (Ruocco et al., in press) and it was isolated as free-living green-brownish aggregates from *P. oceanica* leaves. Despite it was demonstrated that a wide number of cyanobacterium species are able to live in association with taxonomically diverse organisms, such as animals, plants, fungi, algae etc. (Meeks and Elhai, 2002; West and Adams, 1997), *Halomicronema metazoicum* is the only species of this genus that was reported as free-living cyanobacterium in the marine environment. Aqueous extracts of *Halomicronema* sp., isolated from sponges, were able to induce lysis in human erythrocytes and to interfere sea-urchin normal development (Pagliara and Caroppo, 2010) and these data suggest that in the hydrophilic fraction of the *Halomicronema* spent medium there are compounds able to interfere with growth factors. We can speculate that the absence of contaminating and foreign organisms in our culture was due to toxic bioactive compounds, with allelopathic effects, produced by our cyanobacteria strain and released constitutively in the medium as granules as demonstrated by SEM microscopy (Dias et al., 2017).

The growth of fragments of *H. metazoicum*, checked each 20 days, showed daily increment of 2.3% (± 0.3) and 3.3 % (± 1.0) at 20 and 40 days, respectively and an initial lag phase was observed. It has been demonstrated that species of *Phormidium* group that this group of cyanobacteria can use both inorganic and organic phosphate (Pintner and Provasoli, 1958) and some species are able to store phosphates as polyphosphates in volutine granules (Kromkamp, 1987). In addition, that they are not able to use atmospheric nitrogen and must rely, for the growth, on nitrate and ammonia available in the medium, both considered a good source of nitrogen (Fujimoto et al., 1997; Pintner and Provasoli, 1958).

Our results indicate that there was no effect on survival of *A. salina* nauplii in replicates threated with cyanobacteria homogenates. Consequently, our strain does not produce any wound activated, anti-grazing compound active or with detrimental effects on *A. salina nauplii*. Indeed, wound activated compounds, commonly produced as defensive system in diatoms (Maibam et al., 2014; Maibam, 2012; Pohnert, 2000), are rare and (or) poor studied in cyanobacteria and few studies focalized the attention on volatile wound activated infochemicals rather than toxic compounds (Fink et al., 2006a).

The effects on *A. salina nauplii* administrated with spent medium testified a strong toxicity of *H. metazoicum* constitutive produced molecules. *Phormidium-like* cyanobacteria are a common source of new active compounds, such as phycobiliprotein (Madamwar et al., 2015). We observed toxicity after 5 minutes, in the replicates where we administrated the higher concentration of compounds (dilution 1:5). The compound is very active and, after 24 hours, even at intermediate concentration (dilution 1:100). It is impossible to quantify the real concentration of active compound(s) in our treatments, however, other *Phormidium-like* strains produce high active compounds, with apoptogenic and cytotoxic activity, even at low concentrations (Madamwar et al., 2015; Li

et al., 2014). The toxicity observed in spent medium treatment and in 5-minutes treatments suggests an acute effect of administrated active compounds and a reduction of mortality due to the removal of the toxic compounds after 5 minutes from the start of the experiment.

The same compounds were active on the model rotifer (Preston and Snell, 2001) *Brachionus plicatilis* at a concentration comprised between 1:10 and 1:5. The effects were slightly increasing upon time but in most treatments they were evident after 24 h, indicating an acute toxicity clearly affecting the vitality and the survival of tested organisms. Trends of toxicity were consistent among treatments in various times and we chose to take into account the final readings at 24 hours, to simplify the evaluation of the median lethal concentration (Dahms and Hellio, 2009).

The results of toxicity tests performed on rotifers are quite reproducible and the differences among replicates are generally low (Suga et al., 2007). However, the patterns of responses according to salinity are puzzling, when various irradiances and temperatures are considered. In general, the maximum efficacy was observed at intermediate irradiance (140 μE) and higher temperatures (18-22 °C) and salinity (40-44). However, the highest salinity was consistently effective at lower (80 μE) irradiance while intermediate salinity (40) was effective mainly at the highest irradiance (200 μE). According to the trends disclosed by the tests in various conditions, performed on *B. plicatilis*, the production of toxic exudates moderately increases with the temperature, the irradiance and the salinity and it is maximum at 22 °C, 140-200 μE and salinity 44, producing total mortality at a concentration of 1:10. The temperature is a critical factor because the toxicity is lowest in cultures cultivated at 16 °C. Interestingly, at the normal salinity of the Mediterranean (36) the highest irradiance (200 μE) induces a decrease in the toxicity of cyanobacteria. However, the result of these acute toxicology tests measure

the effects of given compounds in specific experimental conditions (Preston et al., 1999) and we cannot exclude that various families of bioactive compounds, having different effects over longer times of exposure, are produced by the same strain.

For this reason it is useful to compare the results with those obtained in standard toxicity tests performed on sea urchin embryos (Romano, 2003). In this case, toxic compounds appear to affect selected phases of embryo development. The first division is hardly affected by the presence of cyanobacteria toxins at a concentration comprised between 1:10,000 and 1:1,000 with no reference to salinity, irradiance or temperature. In fact, all experimental conditions produced high percentages of divided embryos at 1:100,000 and total block of embryo development at 1:1,000. A slight increase of toxicity was observed at the highest temperature (22 °C) and the highest salinity (44), coherently to what observed in *B. plicatilis* tests, but the differences in this case are scarcely significant. Similar trends were observed in the influences on the gastrulation process, since the strongest effects were triggered by the highest temperature (22 °C), especially when coupled with intermediate and highest salinities (40-44) and low or medium irradiances (80-140 μ E). Thus, a different class of compounds could be responsible for this activity, or the changed metabolism of embryos in this phase could be influenced by different compounds, in the range of metabolites produced by cyanobacteria. The patterns were totally inverted when the development of plutei was considered. In this case, in fact, the lowest percentages of normal plutei were triggered by spent medium collected at the lowest temperature (16 °C) without any difference due to irradiance or salinity. In contrast, the highest temperature (22 °C) triggered similar effects only at the highest (44) or intermediate (40) salinity, in accordance with the data obtained with *B. plicatilis*. Thus, we conclude that the compounds influencing the development of larvae, mainly produced at low temperatures, are different from those influencing the mortality of *B. plicatilis* and

the development of sea urchin until the gastrula stage, mainly produced at the highest temperatures and intermediate or low irradiances. Finally, the first division of sea urchin embryos appears as a quite delicate phase, blocked at the same rate by compounds produced in any condition of light, temperature and salinity. Interestingly, the median lethal concentration (Farris et al., 1992) of allelopathic compounds produced at higher temperature (22 °C), medium irradiance (140 µE) and medium-high salinities, was different in *B. plicatilis* and sea urchin embryos. The latter reacted at concentrations of the spent medium at least 2 orders of magnitude lower than those active on rotifers. A second class of compounds could be produced at low temperature (16 °C) without distinction of irradiance and salinity, and it influenced the process of development of sea urchin plutei at very low concentrations, comprised between 1:10,000 and 1:1,000. These low-temperature compounds influenced *B. plicatilis* at such high concentrations as 1:5. Their relatively high median lethal concentration may be easily explained considering that the responsible compounds are released into the spent medium and that a diluted spent medium was used. Once fractioned and identified, the active compounds presently dissolved in the *f/2* medium could reveal their activity at low concentrations.

Proteins and polypeptides (Zhang et al., 2011) are among the most important toxic compounds produced by cyanobacteria and it is known that changes in environmental conditions (e.g. salinity, pH, temperature), modify protein structure and may cause the formation of cytotoxic protein aggregates, with the synthesis of “stress” proteins (Gross, 2004). Among the most interesting compounds produced and released by *Phormidium*-like cyanobacteria are, for example, the portoamides, cyclic peptides having a clear allelopathic activity and an antiproliferative effect on human lung-carcinoma cells (Ribeiro et al., 2017). Consequently, the differences observed at different salinities and temperatures are in line with previous research on cyanobacteria (Chorus et al., 2000)

and indicate that cyclic polypeptides (e.g. microcystins and portoamides), typically produced by these organisms, could be the main products influencing the survival of different model organisms, at different median lethal concentrations.

In addition, some families of microcystins are known to induce apoptosis in various organisms and these compounds, produced at low temperatures, could be responsible for the irregular development of plutei. On the other side, microcystins can trigger cytoskeleton disruption in human hepatocytes (Falconer and Yeung, 1992), after disorganization of cytoplasmic microtubules, cytokeratin intermediate filaments and actin microfilaments (Ding et al., 2000). They were demonstrated to produce oxidative stress, exposing the cells to the activity of reactive oxygen species (ROS). For example, the exposure to microcystins causes oxidative stress in rat Sertoli cells, through decreased antioxidative enzyme activity and increased ROS activity (Yi et al., 2011). Oxidative stress and apoptosis are related processes and the production of ROS has been suggested to be involved in programmed cell death under various conditions, including chemical injury (Kannan and Jain, 2000; Tan et al., 1998; Buttke and Sandstrom, 1994). Thus, these compounds, produced at higher temperatures and irradiances, could be responsible for the block of cell divisions in sea urchin embryos.

These compounds might reveal interesting biotechnological applications even in the field of human medicine (Thompson, 1995) since they might have antitumor activity against some types of tumour cell lines. Different tissues demonstrated variable responses to microcystins (Yi et al., 2011) and a study (Zhang et al., 2011) demonstrated that the expression level of p53 increased when human Sertoli cells were exposed to microcystins, suggesting that they induce apoptosis by modulating the expression of p53, as well as modulating the expression of Bcl-2 proteins. Hence, some of the putative compounds responsible for the observed toxic effects play key roles in various mechanisms involved

in the apoptotic pathways. In particular, p53, bcl-2, bax and caspase-3 are probably involved in cyanobacteria-induced cell damage and toxicity (Dias et al., 2017), and further investigations on the toxicological role of cyanobacterial products in apoptosis-related signaling pathways (Ribeiro et al., 2017) will clarify the nature, the specificity and the mechanism of action of the compounds produced at low and high temperatures by *Halomicronema metazoicum*.

4 : PLANT-ANIMAL INTERACTION

4.1 VOLATILE INFOCHEMICAL PRODUCTION AND PERCEPTION IS AFFECTED BY OCEAN ACIDIFICATION.

4.1.1 ABSTRACT

Communications among marine organisms are widely based on the production, transmission and interpretation of chemical cues. Some volatile organic compounds (VOC) can play the role of infochemicals and they can be interpreted differently by target organisms according to a wide range of chemical, physical and biological variables. Ocean acidification can alter the production in the source organism, as well as the interpretation in target organisms, of infochemicals.

Two diatoms (*Cocconeis scutellum parva* and *Diploneis* sp.) and a macroalgae (*Enteromorpha prolifera*), epiphyte of *P. oceanica* leaf, were isolated and cultured at two pH conditions (pH 8.2 and pH 7.7), the biomass collected and the associated VOCs extracted. An odour choice experiment was performed on such *P. oceanica* associated invertebrate species, as *Alvania lineata*, *Rissoa italiensis*, *Hippolyte inermis* and *Idotea balthica*. For the last two species, tests were performed both on adults and juveniles. Volatile organic compounds produced by algae cultured at normal or acidified pH were tested at the same pH on target organisms. A complex pattern of behaviour has been identified in response to VOCs produced by algae. Differences in the behaviour in associated vagile fauna according to the species, the VOCs concentration as well as the pH conditions have been identified.

4.1.2 INTRODUCTION

Marine organisms use chemical cues to interact and communicate (Zupo and Maibam, 2011; Pohnert et al., 2007; Brönmark and Hansson, 2000) in an environment suffused with information produced by prey, predators, competitors or simply by conspecific individuals. This communication has evolved in bacteria, plants and animals as biological machinery to sense and process such information (Vos et al., 2006). Infochemical, as a term, refers to compounds that bring information that can be received and interpreted by various species living in the environment (Vet and Dicke, 1992).

Various infochemicals trigger different specie-specific reactions according to their role in the food webs (Zupo et al., 2016). Among infochemicals, volatile organic compounds (VOCs) are low-weight compounds with a high structural diversity in aquatic ecosystems, that are transferred among organisms, acting as kairomones (interspecific communication), pheromones (intraspecific communication) or as activated defences (Fink, 2007; Dicke and Sabelis, 1988). Volatile infochemicals can be interpreted by organisms as warnings, indicating the presence of a predator or a toxic compound, triggering an avoidance or escape reaction, or can be interpreted as an attractive signal, indicating for example a food source, mate or host location (Zupo et al., 2016; Jüttner et al., 2010). The information is carried by the characteristics of the perceived molecules and by the concentration of infochemicals diffused in the water; this means that various concentrations of the same VOC could trigger different reactions (Zupo et al., 2015) in target organisms. Co-evolutionary constraints, for example, cause that invertebrates associated to seagrass meadow are a) able to identify native “odour”, normally present in their specific habitat towards a bouquet of various VOCs, and b) unable to identify and interpret “odours” typically produced by organisms living in a completely different habitat (Jüttner et al., 2010).

Ocean acidification (OA) deeply affects the ecology of oceans and a wide range of consequences may be forecasted. OA increased in the last decades due to anthropogenic CO₂ emissions (Diaz-Pulido et al., 2016) and a future decrease of pH, in the order of 0.5 points, is forecasted to be realized by the end of the present century (Turley et al., 2006; Raven et al., 2005). The rising of atmospheric carbon dioxide will cause changes in the chemistry of oceans and it will affect biological processes, such as physiological processes and metabolisms, influencing the production of secondary metabolites, VOCs and other infochemicals (Poore et al., 2013), the molecular assemblages of informative molecules and the receptors functionality to recognize them (Maibam et al., 2015) and the chemotaxis among organisms, with consequences on the plant-animal interaction and of community associations (Knutzen, 1981) in aquatic environments.

A change in secondary metabolites produced by algae upon wounding may play a key role in shaping trophic webs and in the small scale structure of the temporary and spatial distribution of invertebrates (Vos et al., 2006). Several organisms evolved the ability to recognize VOCs generated during the wounding activity, using them to understand what is happening in the surrounding environment and to take important decisions (Fink, 2007) but the perception of these infochemicals are altered in an acidified marine environment (Zupo et al., 2015; Dixon et al., 2010; Hall-Spencer et al., 2008). Metabolic alteration due to high concentration of CO₂ changes the plant-animal communications and cause a misunderstanding in the interpretation of the information as it was previously demonstrated both in benthic (Zupo et al., 2015, 2016) and in planktonic organisms (Maibam et al., 2015; Havas and Rosseland, 1995). In parallel, algae living in an acidified environment may modify their patterns of production of secondary metabolites (some of them play the role of infochemicals) and this additional factor should be taken in consideration to understand “wrong” responses in target organisms.

To understand these fundamental aspects within the complex ecology of *P. oceanica* meadows and the relationship among VOCs, vagile fauna and seawater acidification, we investigated the behavioural responses, at normal and acidified pH, of *Hippolyte inermis* (adults and juveniles), *Idotea balthica* (adults and juveniles) and two species of *Rissoidea* family, *Alvania lineata* and *Rissoa italiensis*, to volatile infochemicals produced by three epiphytes of *P. oceanica* (*Cocconeis scutellum parva*, *Diploneis* sp. and *Enteromorpha prolifera*) cultured at normal (8.2) and acidified pH (7.7). These three epiphytes of *P. oceanica* leaves are also an important diet component of the selected target organisms (Leidenberger et al., 2012; Zupo et al., 2007; Naylor, 1955).

Hippolyte inermis, a decapod living in *Posidonia oceanica*, takes advantage of an apoptogenic compound produced by diatoms of genus *Cocconeis* to shift its sex in some seasons (Zupo et al., 2014; Zupo, 2000). It is a well-studied model to understand plant-animal interactions, apoptosis and chemical ecology (Matalipassi, Maibam, et al., 2018; Zupo et al., 2016; Zupo, 2001, 1994). In *H. inermis*, sex reversal is a fundamental mechanism to stabilise natural populations. The apoptosis of the androgenic gland induces the destruction of the testis and triggers the production of early females, increasing the reproductive fitness of the species (Zupo and Messina, 2007). It has been demonstrated that *H. inermis* is able to detect and recognize volatile compounds, produced by epiphytes living in their typical habitat, probably due to co-evolutionary processes (Maibam et al., 2014; Fink, 2007).

The euryhaline crustacean isopod *Idotea balthica* exhibits an almost cosmopolitan distribution; it is very abundant in littoral and sublittoral tidal shores (Leidenberger et al., 2012; Sturaro et al., 2010; Casagrande et al., 2006; Orav-Kotta and Kotta, 2004). In such areas as the brackish waters of the Baltic sea, it is considered among the most important necto-benthic herbivore (Schaffelke et al., 1995). *Idotea balthica* is omnivorous, eating

benthic micro algae, filamentous algae, macroalgae, detritus, epiphytes and small invertebrates as well as its conspecifics (Bell and Sotka, 2012; Leidenberger et al., 2012; Sturaro et al., 2010; Jormalainen et al., 2001; Merilaita and Jormalainen, 2000). In fact, these marine isopods ingest a considerable quantities of *P. oceanica* dead leaves, a food characterized by a high fibre content, and encrusting epiphytes, such as animals, micro and macroalgae, playing a key role in the degradation process of *P. oceanica* litter (Wittmann, Mazzella, et al., 1981; Wittmann, Scipione, et al., 1981). In idoteids, the grazing activity is possible due to the presence of large molar processes in each mandible and heavily chitinized structures for crushing, biting or scraping the food material (Naylor, 1955).

Rissoidae are an important family of mollusks within the class Gastropoda, with 28 genera and 318 species. They are mainly marine organisms and represent an important component of the food chain being nourishment to the other benthic invertebrates, birds and demersal fishes (Warén, 1996). They are considered herbivores-deposit feeders, with *Rissoa italiensis* typical of shallow bed at the depths of 1-3 metres (Gambi et al., 1992) and *Alvania lineata* distributed at intermediate depths from 3 to 10 metres (Milazzo et al., 2000).

The present study aimed at checking: 1) if micro and macro algae will change their production of VOCs in an acidified world and 2) if OA will modify the communication in associated and coevolved species.

4.1.3 MATERIALS AND METHODS

4.1.3.1 COLLECTION AND TREATMENT OF ALIVE SPECIES

Benthic invertebrates (vagile organisms) were collected in a seagrass meadow off Lacco Ameno d'Ischia, in the Gulf of Naples (Italy) by horizontally towing a plankton trawl horizontally on the surface of *Posidonia oceanica* leaves. Samples were immediately sorted on boat to separate the main taxa. Interesting species were identified in the laboratory, separated alive and divided according to the size. Organisms were reared in 2-L aerated vessels containing 1.5 L of filtered seawater, and they were fed on epiphytized leaves of *P. oceanica*. Animals were starved 24 hours prior to the start of choice experiments. In addition, individuals tested in acidified condition tests were slowly adapted to the pH regime.

Seagrass leaves were collected at the same collection site. Epiphytes were isolated under a stereomicroscopy and thalli of *Enteromorpha prolifera* were detached using forceps. Macroalgal thalli were transferred to sterilized Petri dishes and cultured in Guillard's *f/2* medium without silica (Sigma-Aldrich, Milan, Italy). They were examined every 7 days and other epiphytes were removed prior to re-inoculating cultures. When the cultures appeared clean of other organisms, we checked the axenic condition of cultures under SEM microscopy.

Two benthic diatoms were taken into consideration: *Cocconeis scutellum parva* and *Diploneis* sp. Diatoms were cultured in continuous axenic culture in multi-well filled with Guillard's *f/2* medium with silica (Sigma-Aldrich, Milan, Italy). Micro and macroalgae mother cultures were kept under controlled conditions (18 °C, 12/12h photoperiod) in a thermostatic chamber.

4.1.3.2 SET-UP OF NEWLY DEVELOPED BIOREACTORS

Innovative photo-bioreactors were set to culture *E. prolifera* and the two benthic diatoms, either in normal or in acidified conditions (Fig. 4.1). Photobioreactors were constructed using Pyrex dishes (300 mm L x 200 mm W x 40 mm H) covered with a heat resistant glass. The cover had a narrow opening where a pH probe (InLab®Micro pH, Mettler Toledo) was introduced. The InLab Micro is a probe designed to work in minimum sample volume of 3 millimetres; it is made in steel and specifically built for microbiological applications. The pH probe was connected, via BNC cable, to a pH controller (SMS122, Milwaukee) set at pH 8.2 for normal condition cultures and at pH 7.7 for acidified condition cultures. According to the pH, the controller switched on and off the electronic valve associated to the CO₂ regulator (CO₂ Energy, Ferplast). CO₂ was insufflated in the photo-bioreactor using a plastic stripette, firmly fixed to a second narrow opening in the glass cover. In addition, a centrifuge pump (Askoll Pure pump 300) was used to avoid water stratification and presence of a pH gradient. Microcontrollers (EnerGenie EG-PMS2-LAN) were used to cyclically activate the centrifuge pump (1 minute on, 29 minutes off). Photo-bioreactors have a total volume of 2.4 litres and they were filled with 1.6 litres of medium.

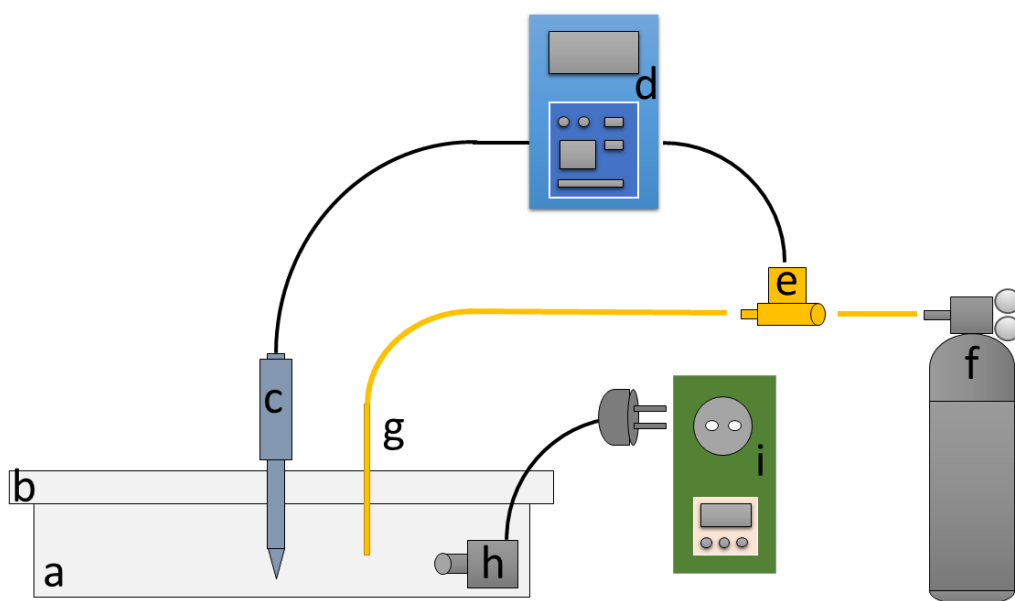


Fig. 4.1: Photobioreactors devised to culture benthic algae at normal and acidified pH. Each reactor was constructed using a Pyrex dish (a) covered with a heat resistant glass plate (b). In the opening at the centre of the cover was housed a pH probe (InLab®Micro pH, Mettler Toledo; c). The pH probe was connected to a pH controller (SMS122, Milwaukee; d) that controls an electronic valve (e). The electronic valve is connected, on one side, to the CO₂ regulator (CO₂ Energy, Ferplast; f), and on the other side to a plastic stripette (g), fixed in a secondary opening in the glass cover. A centrifuge pump (Askoll Pure pump 300; h) was placed on one side of the photobioreactor to avoid water stratification and was temporized by a microcontroller (EnerGenie EG-PMS2-LAN; i).

4.1.3.3 EXTRACTION OF ODOURS AND PREPARATION OF AGAROSE GELS

Diatoms were collected from 6 photobioreactors by scraping their surface with a steel blade. The material collected in each Petri dish was washed out using 100 ml of filtered seawater and transferred into a clean Erlenmeyer flask. The biomass collected from 6 Petri was then centrifuged (10 min at 1500 g at 4 °C) and the supernatant was discharged.

The vessels containing the diatom biomass were immediately frozen (-20 °C) up to their transfer to another laboratory for the extraction VOCs and for chromatographic analyses. Macroalgae were cultivated in the above-described photobioreactors. At the end of the grow-out period they were collected by clean forceps, the culture liquid was dropped out and the biomass produced was transferred into 50 ml tubes and immediately frozen (-20 °C) up to their transfer to another laboratory for the extraction VOCs and the chromatographic analysis.

To simulate wounding and production of odours, frozen algal pellets (500 mg FW for each alga) were transferred into 100 ml flasks and activated by thawing them in 25% NaCl (Fink et al., 2006b). Cells crushing led to activation of lipoxygenase cascade and to the production of VOCs (Jüttner, 2005). Closed-loop stripping (45 minutes at 22°C) was used to concentrate VOCs and samples were then absorbed on a Tennax TA cartridge. Elution of the Tennax cartridge was performed using 6 millilitres of diethyl-ether, the eluates was collected in glass tube, ether evaporated gently in a stream of nitrogen gas (N₂, grade 5.0) and the residue was re-dissolved in 70 microliters of pure ethanol. Controls were obtained using the same procedure but using filtered culture medium not containing any algae. The produced VOCs solutions were stored at -80°C.

4.1.3.4 CHROMATOGRAMS

Samples of our microalgae were sent to Professor Patrik Fink of the University of Cologne to perform biochemical analysis to investigate differences in the Volatile Organic Compounds productions. Analyses are still uncompleted but final data, important to completely interpret the results of bioassays herein reported, will be available in the next months, ready for publication of results.

4.1.3.5 PREPARATION OF STOCK SOLUTIONS AND GELS

We dissolved 1.2 g of agarose (Sigma A-9045) in 200 mL of filtered and sterilized seawater to obtain a 0.06% agarose gel. The agarose solution was heated (80 °C), mixed until transparent and we added 3.3 mL of 0.1 M NaOH to adjust the pH to a value of 8.2. Control gel and three VOCs gels were prepared including 0.5, 5, 50 µl of, respectively, control and VOCs ethanol solution into the agar solutions close to the room temperature, just before gelling (Zupo et al., 2016). The obtained control and the three VOCs solutions, at low, medium and high concentration, were poured into Petri dishes and allowed to gel in refrigerator at 5 °C for 2 hours prior to be used into the assays. Gels were cut into blocks of 1 cm³ (using clean glass coverslips) prior to be offered to the animals in the static choice experimental arena (Jüttner et al., 2010).

4.1.3.6 TESTS ON INVERTEBRATES

Odour choice tests were performed to test the effect of plant bouquets of odours on *H. inermis*, *I. balthica*, *A. lineata* and *R. italiensis*. We performed two different tests: a first set using static choice experimental arena, as described in Jüttner et al. (2010); a second in dynamic flow-through flume system, as described by Atema et al. (2002) in order to compare the chemotactic reactions of organisms according to different experimental scenarios.

a) Static arenas

Experimental arenas were obtained into Petri dishes (14 centimetres diameter) each containing 200 mL of filtered seawater (approx. 1 cm of water in each Petri). Experiments were performed in a room at 18°C with shaded light source, in order to minimize

phototactic reactions biasing the results. Petri dishes were subdivided into 5 sectors corresponding to various concentrations of the VOCs diffused by simple elution.

Five individuals were positioned at the centre of each arena at the start of an experimental trial. The movements of test subjects were recorded every 5, 10 and 15 min., by visually checking the number of individuals in each sector.

b) Flow-through flumes

Straight flow-through flume system (Atema et al., 2002; Voigt and Atema, 1996) were constructed and used to test the effect of VOCs dissolved in the water flow at a low speed. They were made of choice systems able to keep two parallel, not mixing, flumes (Kroon, 2011). The flume systems were built using grey glasses to avoid influences of light on the behavioural choices. They were divided by two grids (0.1 mesh) in a straws area, an experimental arena and an overflow area. The experimental arena had a length of 8 cm by 14 cm (width). Water level was 3 centimetres and the total volume was 900 millilitres. The result was a non-mixing flow of water, on one side, containing the VOCs and on the other side containing filtered seawater. Two water containers (250 ml) were positioned on the two opposite corners of the straws areas and water streams were kept divided and parallel by plastic straws fixed over the bottom. They were filled with stock solutions, represented by filtered and sterilized seawater with 0.5, 5, 50 μl of, respectively, control and VOCs ethanol solution. The water was discharged by an overflow placed at the other side of the flumes.

The velocity of the water flow was 5 cm . min⁻¹; at this flux speed the containers were emptied in 15 min from the start of each test. Invertebrates could move freely and eventually choose one sides of the container, interested by a specific flume, containing by the VOCs (test) or not (control).

Six individuals, for each species, age and for each treatment were tested in 8 replicates. The position of test and control targets were inverted in each test to avoid the influence of external influences. Just before the start of each test the VOCs containers were filled up with either the appropriate VOCs (+) or the control (-) stock solutions. At the start of experiments the test animals were placed in a central rectangular zone. The number of individuals present in each side of the experimental arena was recorded at 5, 10 and 15 minutes.

4.1.3.7 STATISTICAL ANALYSIS

The significance of differences in the distribution of individuals between the positive (VOC areas) and negative (control areas) in 8 trials for each test by repeated-measures ANOVA with Prism 7 (Graph-pad software). In each sector, the mean number of individuals was calculated for each test in the experimental time. An attraction-repulsion (A/R) index was calculated for each test according to the following equation (Jüttner et al., 2010):

$$\frac{A}{R} \text{ index in static arenas} = N^{\circ} \text{Ind}_2 + N^{\circ} \frac{\text{Ind}_1}{2} - N^{\circ} \text{Ind}_{-2} + N^{\circ} \frac{\text{Ind}_{-1}}{2}$$

$$\frac{A}{R} \text{ index in flow - through flumes} = N^{\circ} \text{Ind}_2 - N^{\circ} \text{Ind}_{-2}$$

where $N^{\circ} \text{Ind}_2$, $N^{\circ} \text{Ind}_1$, $N^{\circ} \text{Ind}_{-2}$, $N^{\circ} \text{Ind}_{-1}$ is the number of individuals in sector +2, +1, -2, -1 respectively.

The attraction-repulsion index, in each time-laps, was used to calculate the linear regression for each test and the slope of each equation represents the tendency of each species in each condition to move towards positive (value +2), negative (value +1) or neutral areas (value 0). The slopes of each linear regression equation indicates the tendency to move towards a target but not the time spent by individuals in each sector and, in order to determine in which sector were organisms during the experimental time,

we calculated a second index, called integral time (INT), according to the following equation (Jüttner et al., 2010):

$$INT = \sum (T_1 + T_2 + T_3 + T_4)$$

where T_1 , T_2 , T_3 , T_4 are the attraction-repulsion value for each time laps. A positive integer score indicates a higher permanence in the positive sectors; negative values indicate a higher permanence in negative sectors; values close to 0 indicate that individuals are not moving or a random movement in the two directions.

The scores obtained for the attraction-repulsion index and for the integral time, for each test, were organized in an orthogonal coordinate system to summarize the results. Species with positive integer scores and negative slopes (first sector) indicate a preference for the volatile organic compound but a tendency to move towards the control; species in second sector, with both positive values, were attracted by odour and were moving towards the positive zones. Species in the third sector (positive slope, negative INT) indicates that individuals were moving towards the source of VOCs, but remained most of the time in the negative sectors; and finally, ordering in the fourth sector indicates high repulsive tendency (negative slope, negative INT).

4.1.4 RESULTS

Preliminary results indicated statistical differences in the VOCs production in the two diatoms and few differences in the production of VOCs, by *E. prolifera*, in the two culture conditions (Data still not available).

A complex pattern of chemotactic reactions was shown by our putative model species according to algal species and pH conditions. In addition, in some case, the behavioural reaction to VOCs differed in static arenas and flow-through flumes. *Alvania lineata* (Fig. 4.2) was strongly attracted by of *Cocconeis scutellum parva* VOCs, at low and medium concentrations, cultured and tested at normal pH, strongly repulsed by *Enteromorpha prolifera* VOCs, cultured at normal conditions. Low concentration of *Enteromorpha prolifera* VOCs produced an attraction when cultured and tested in acidified conditions. A contrasting response was triggered by high concentrations of *C. scutellum parva* VOCs (normal conditions), with an attraction observed in static chamber and repulsion in flumes. In addition, differences were seen in the reactions in static and flume chambers to VOCs produced at normal and acidified conditions, such as the score obtained by *C. scutellum parva* and *E. prolifera*. Snails tested in static chambers showed a tendency to move through low concentrations of VOCs produced by *Diploneis* sp., in both conditions, and *Cocconeis scutellum parva*, in both testing conditions.

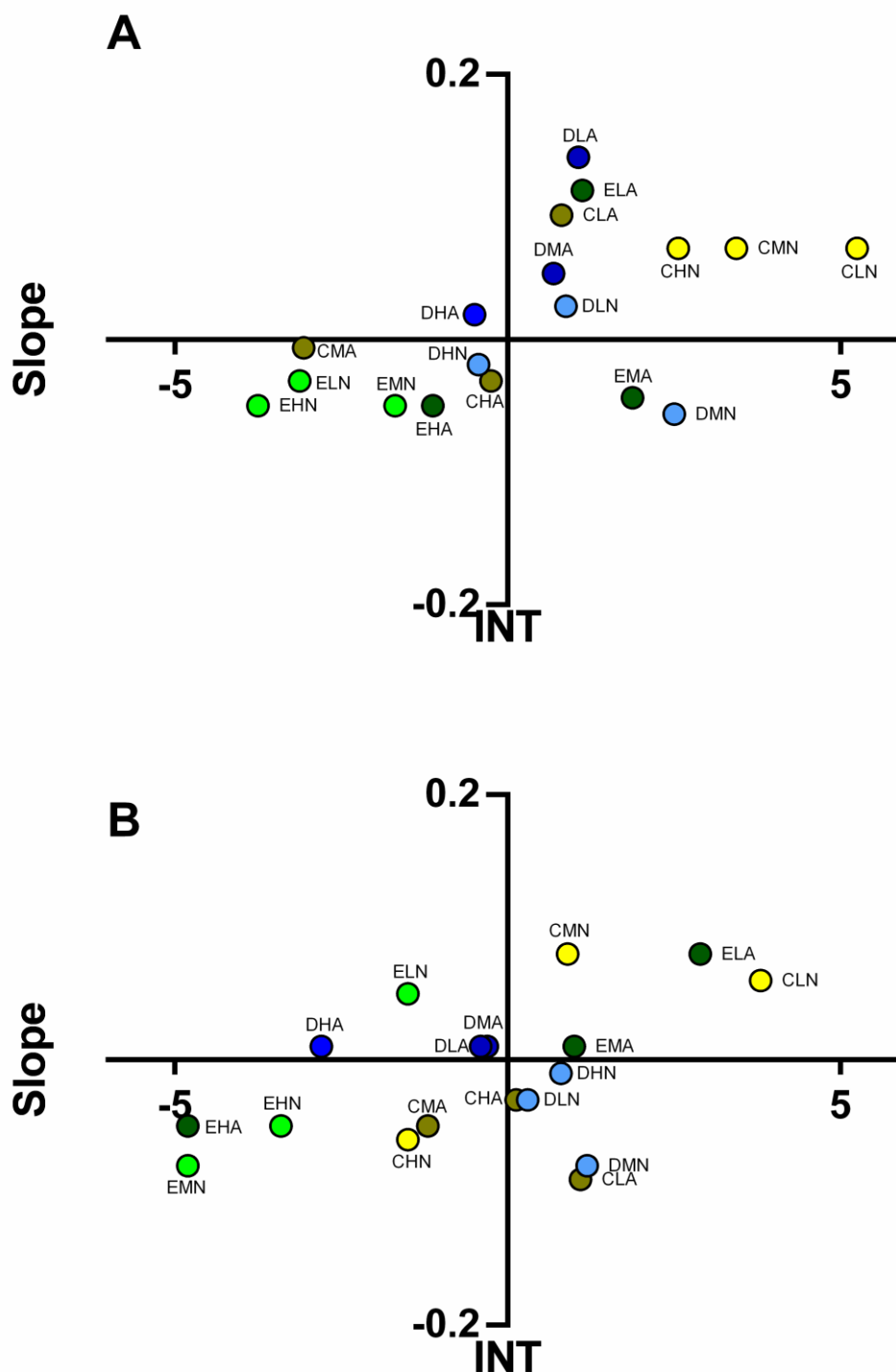


Fig. 4.2: Ordination of the chemotactic reactions of *Alvania lineata*, in static arenas (A) and flow-through flumes (B), according to exposition to Volatile organic compounds produced by 3 algae at 3 concentrations in 2 pH conditions, in the space expressed by Integer (INT) and slope (Slope) indices. The 3-letter codes indicate algae, Volatile organic compound concentration and pH as reported in Table 4.1.

An attractive behaviour was triggered in *Rissoa italiensis* (Fig. 4.3) by acidified *C. scutellum parva* and *Diploneis* sp. VOCs and a repelling one, with differences between static arenas and flow-through flumes, to the same diatoms cultured at normal conditions. *Rissoa italiensis* was repelled by *E. prolifera* VOCs (normal conditions) at high concentration in both odour choice tests and was slightly attracted by normal and high concentrations of *E. prolifera* VOCs cultured at acidified conditions. The response to *E. prolifera* VOCs cultured at normal condition increased linearly: in low concentration VOCs, *R. italiensis* spent most of the experimental time in positive sectors, in medium concentration VOCs, the source of odour was about to be ignored and in high concentration of VOCs a repulsion was observed.

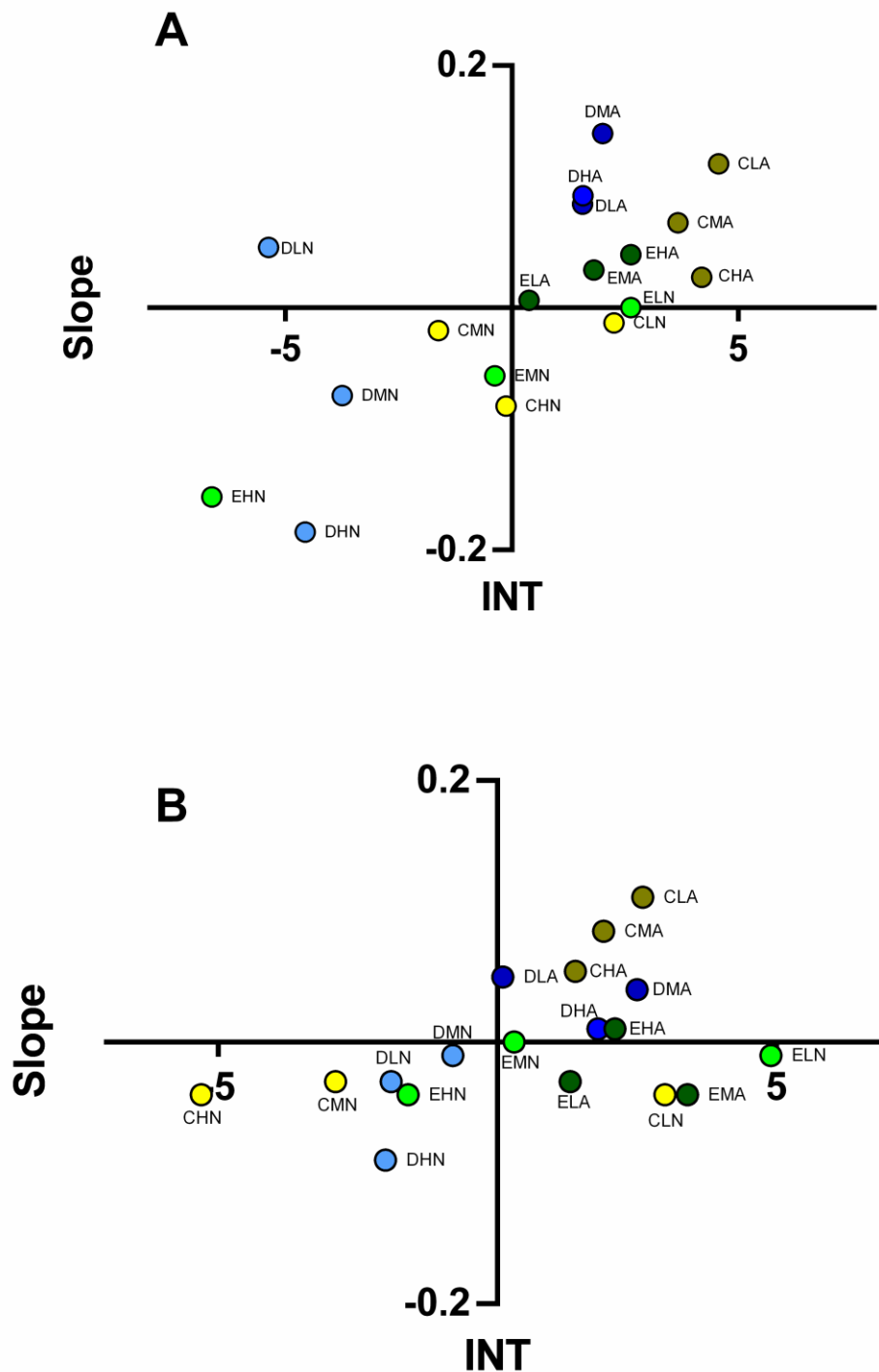


Fig. 4.3: Ordination of the chemotactic reactions of *Rissoa italiensis*, in static arenas (A) and flow-through flumes (B), according to exposition to Volatile organic compounds produced by 3 algae at 3 concentrations in 2 pH conditions, in the space expressed by Integer (INT) and slope (Slope) indices. The 3-letter codes indicate algae, Volatile organic compound concentration and pH as reported in Table 4.1.

Adult individuals of *Hippolyte inermis* (Fig. 4.4) were attracted, with some differences between the two odour choice tests, by low concentration of VOCs produced by *Diploneis* sp. (acidified conditions) and *Enteromorpha prolifera* (both culture and test conditions) and repulsed by higher concentrations of VOCs produced by the same algae as well as by VOCs produced by *Cocconeis scutellum parva* (normal and acidified conditions).

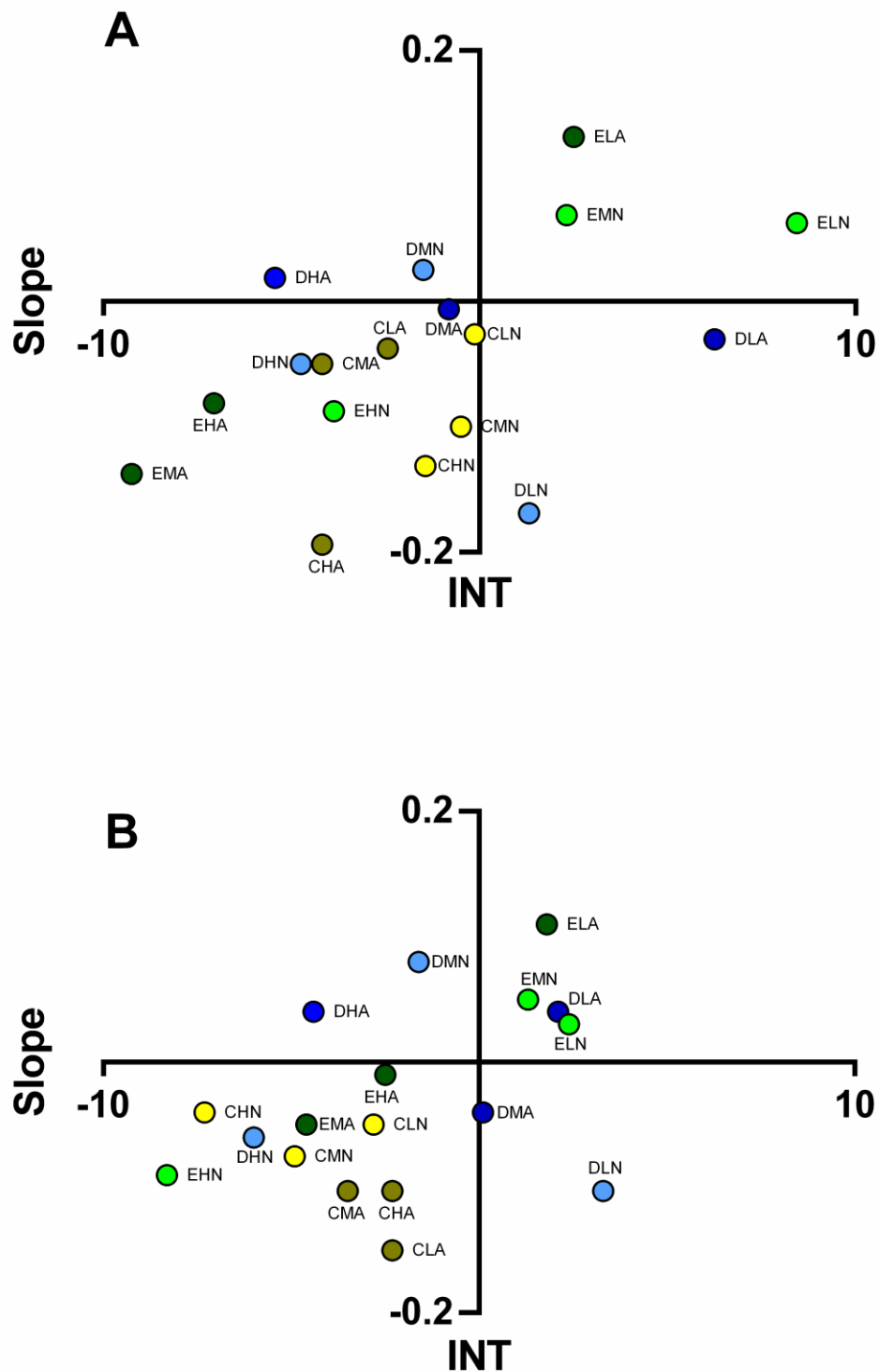


Fig. 4.4: Ordination of the chemotactic reactions of *Hippolyte inermis* (adults individuals), in static arenas (A) and flow-through flumes (B), according to exposition to Volatile organic compounds produced by 3 algae at 3 concentrations in 2 pH conditions, in the space expressed by Integer (INT) and slope (Slope) indices. The 3-letter codes indicate algae, Volatile organic compound concentration and pH as reported in Table 4.1.

In contrast, juveniles *H. inermis* (Fig. 4.5) exhibited contrasting behavioural reactions: they were attracted by *Cocconeis scutellum parva* at low and medium concentrations (normal conditions) and by *Diploneis* sp. (low VOCs concentration, normal conditions). Individuals spent most of the experimental time in positive sectors of the choice arenas in the case of low concentrations *E. prolifera* VOCs (cultured at normal conditions) and move towards but spent most of the experimental time in negative sectors of the choice arenas in the case of low concentrations *Diploneis* sp. VOCs (cultured at acidified condition). High concentrations of VOCs of the three algae at both culture conditions and *Diploneis* sp. and *Enteromorpha prolifera* at medium concentration (normal and acidified conditions) produced a repulsive response in both the odour choice chambers.

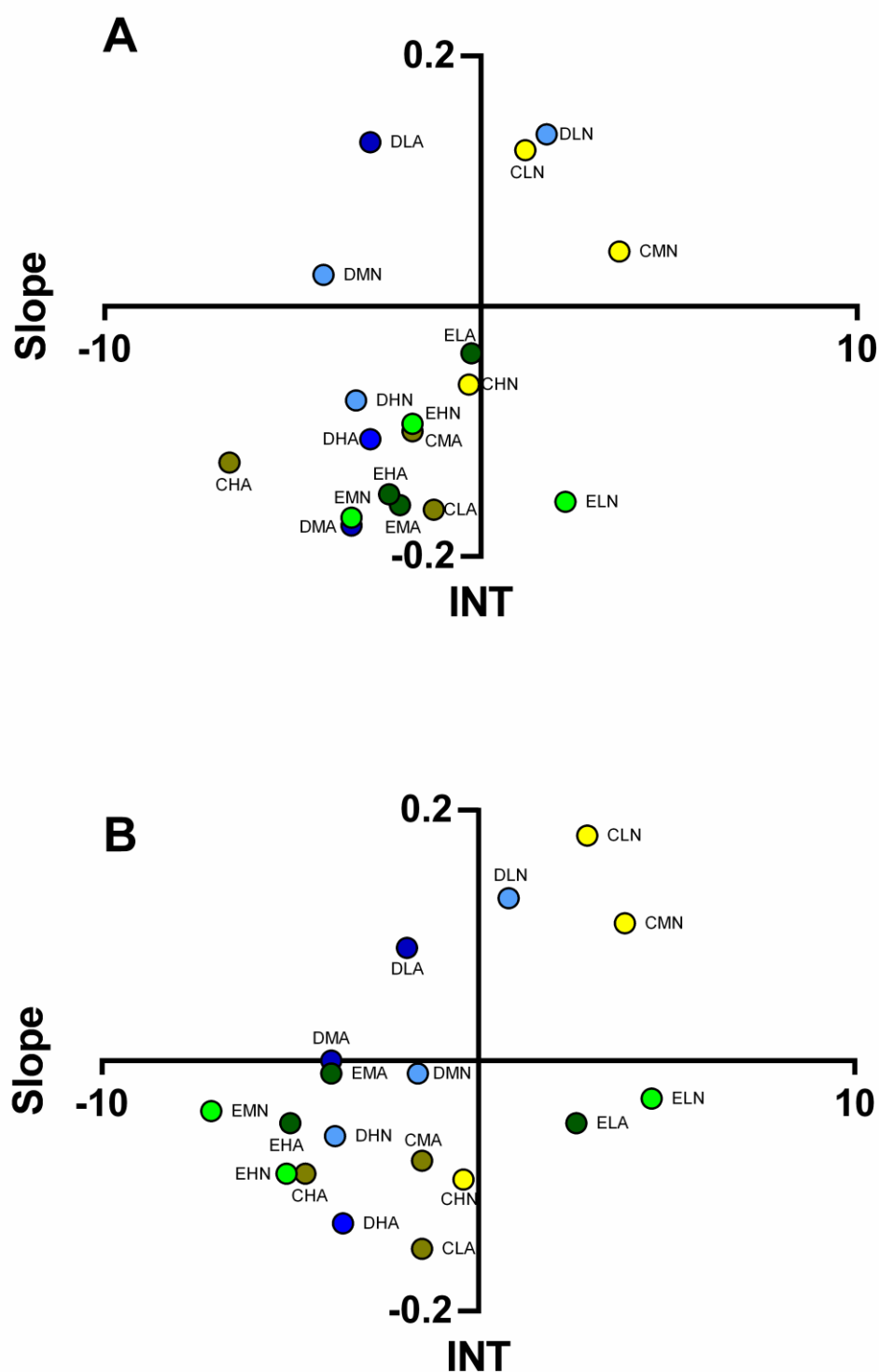


Fig. 4.5: Ordination of the chemotactic reactions of *Hippolyte inermis* (juvenile individuals), in static arenas (A) and flow-through flumes (B), according to exposition to Volatile organic compounds produced by 3 algae at 3 concentrations in 2 pH conditions, in the space expressed by Integer (INT) and slope (Slope) indices. The 3-letter codes indicate algae, Volatile organic compound concentration and pH as reported in Table 4.1.

Adult *I. balthica* (Fig. 4.6) were attracted by VOCs produced by *C. scutellum parva* cultured at acidified pH, in static arenas. In flow-through flumes at the same conditions, adults of *I. balthica* spent most of the experimental time in negative sectors but moving through the positive areas. *Cocconeis scutellum parva* at normal pH (medium and high concentrations) produced, both in odour choice chambers and flumes, repulsive reactions as well as *Diploneis* sp. VOCs at high concentration. VOCs of *E. prolifera* produced a contrasting behavioural reaction: at high concentration, *I. balthica* was not attracted in normal cultured VOCs but they were repelled in acidified conditions. Lower concentrations of *E. prolifera* VOCs produced an attractive reaction.

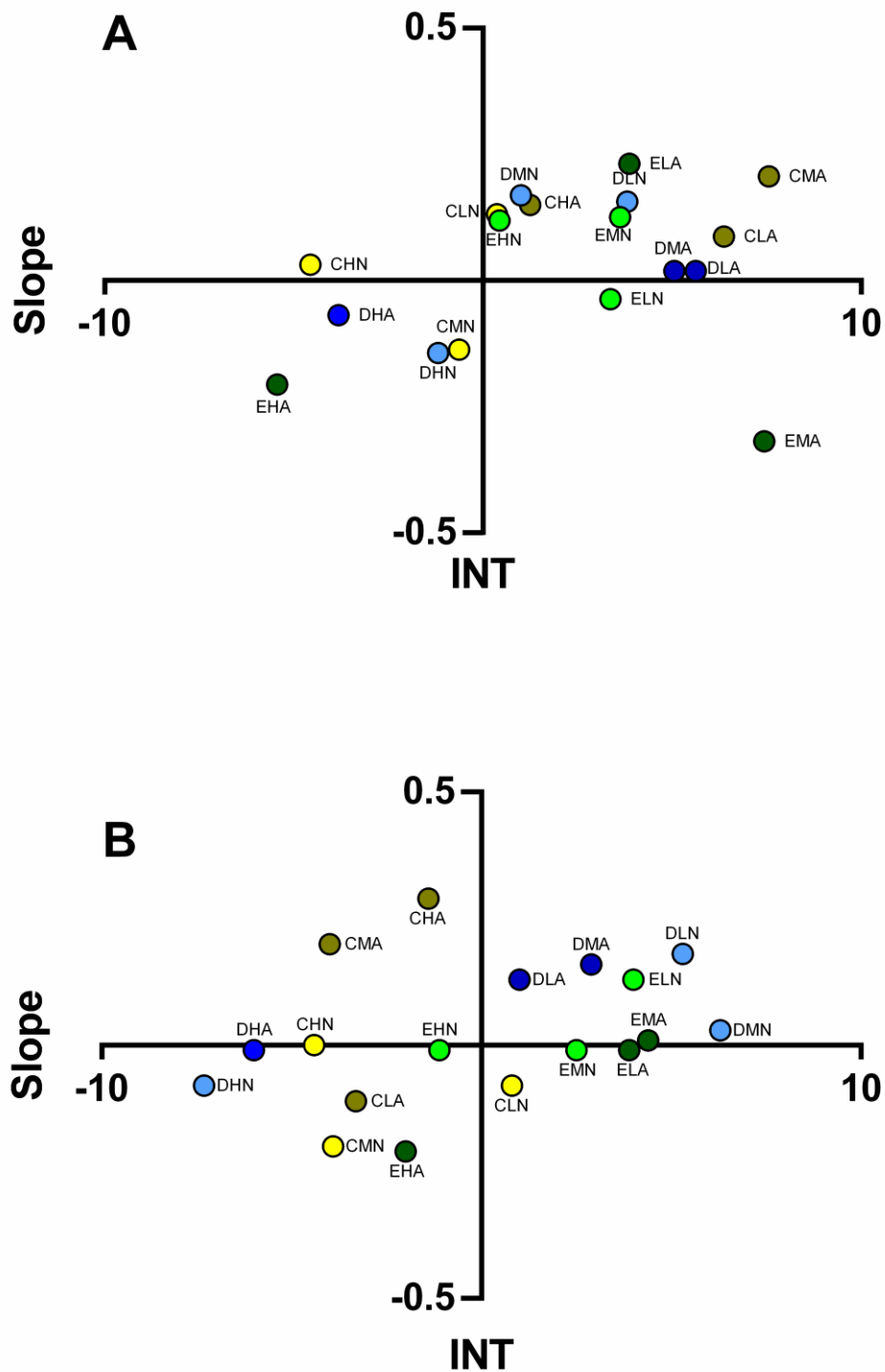


Fig. 4.6: Ordination of the chemotactic reactions of *Idotea balthica* (adult individuals), in static arenas (A) and flow-through flumes (B), according to exposition to Volatile organic compounds produced by 3 algae at 3 concentrations in 2 pH conditions, in the space expressed by Integer (INT) and slope (Slope) indices. The 3-letter codes indicate algae, Volatile organic compound concentration and pH as reported in Table 4.1.

Due to the unique behaviour of *I. balthica* juveniles (Fig. 4.7) it was impossible to perform odour choice experiments in flow-through flumes. Isopods juveniles have a well-developed ability to climb glass walls, plastic nets and to pass below the plastic frames used to divide the different areas of the flume. In static arenas, juveniles of *I. balthica* were attracted by *Cocconeis scutellum parva* cultured at normal pH and repulsed (at high concentration) or almost not interested (medium and low concentration) by VOCs produced by the same alga cultured and tested at acidified conditions. *Enteromorpha prolifera* (normal conditions – medium and high concentration) produced a slightly repulsive reaction. Low and medium concentrations of VOCs produced by acidified *E. prolifera* (acidified conditions) and by *Diploneis* sp. (both conditions) produced persistence in positive sectors and attraction, respectively.

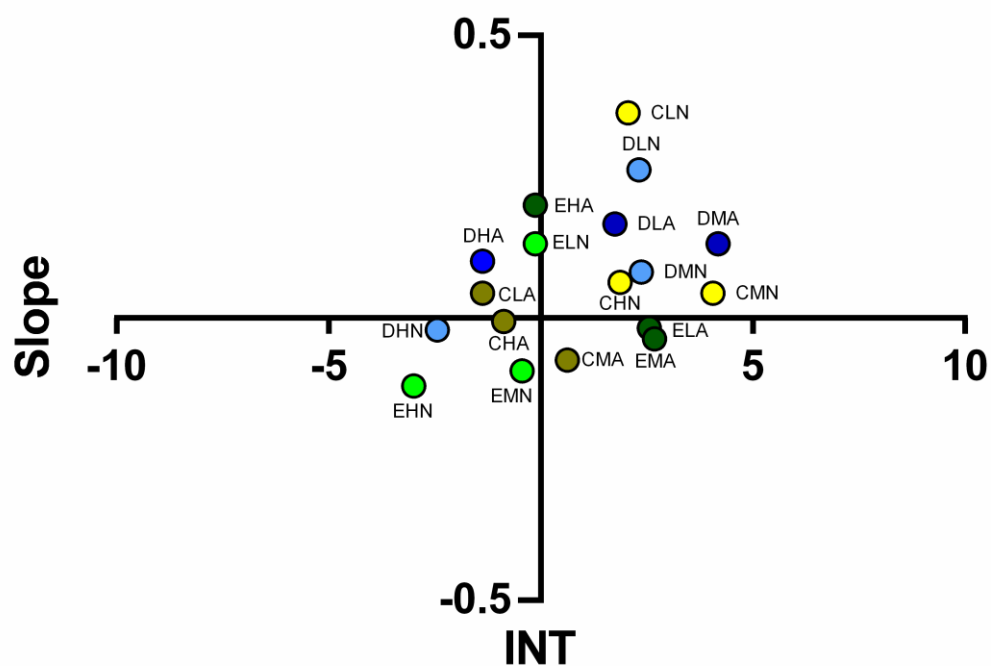


Fig. 4.7: Ordination of the chemotactic reactions of *Idotea balthica* (juvenile individuals) in static arenas according to exposition to Volatile organic compounds produced by 3 algae at 3 concentrations in 2 pH conditions, in the space expressed by Integer (INT) and slope (Slope) indices. The 3-letter codes indicate algae, Volatile organic compound concentration and pH as reported in Table 4.1.

Code	VOC sources	Concentration	Culture and test condition
CLN	<i>Cocconeis scutellum parva</i>	Low: 0.5 µl	Normal pH: 8.2
CMN	<i>Cocconeis scutellum parva</i>	Medium: 5 µl	Normal pH: 8.2
CHN	<i>Cocconeis scutellum parva</i>	High: 50 µl	Normal pH: 8.2
CLA	<i>Cocconeis scutellum parva</i>	Low: 0.5 µl	Acidified pH: 7.7
CMA	<i>Cocconeis scutellum parva</i>	Medium: 5 µl	Acidified pH: 7.7
CHA	<i>Cocconeis scutellum parva</i>	High: 50 µl	Acidified pH: 7.7
DLN	<i>Diploneis</i> sp.	Low: 0.5 µl	Normal pH: 8.2
DMN	<i>Diploneis</i> sp.	Medium: 5 µl	Normal pH: 8.2
DHN	<i>Diploneis</i> sp.	High: 50 µl	Normal pH: 8.2
DLA	<i>Diploneis</i> sp.	Low: 0.5 µl	Acidified pH: 7.7
DMA	<i>Diploneis</i> sp.	Medium: 5 µl	Acidified pH: 7.7
DHA	<i>Diploneis</i> sp.	High: 50 µl	Acidified pH: 7.7
ELN	<i>Enteromorpha prolifera</i>	Low: 0.5 µl	Normal pH: 8.2
EMN	<i>Enteromorpha prolifera</i>	Medium: 5 µl	Normal pH: 8.2
EHN	<i>Enteromorpha prolifera</i>	High: 50 µl	Normal pH: 8.2
ELA	<i>Enteromorpha prolifera</i>	Low: 0.5 µl	Acidified pH: 7.7
EMA	<i>Enteromorpha prolifera</i>	Medium: 5 µl	Acidified pH: 7.7
EHA	<i>Enteromorpha prolifera</i>	High: 50 µl	Acidified pH: 7.7

Table 4.1: Summary of the codes used to identify the different Volatile organic compound sources considered, the concentrations and the culture/testing conditions for each test.

4.1.5 DISCUSSION

Marine ecosystems are suffused with a quantity of infochemicals produced by micro- and macroalgae, prokaryotes, protozoans, vertebrates and invertebrates. It is evident that chemical cues may play a key-role in the ecology of organisms, especially invertebrates, associated to aquatic environments (Derby and Sorensen, 2008). Various factors can influence the production of infochemicals: local ecological conditions and their temporal variations can affect the concentration and the composition of infochemicals produced by micro- and macro algae and, in addition, the same factors can change the response of target organisms due to alteration of the perceptive abilities of individual species of invertebrates (Wyatt et al., 2014). Marine invertebrates rely upon chemical cues to communicate and find food sources and chemical stimuli produce complex behaviour of invertebrates due to individual variability and stochastic factors that can influence the organism's chemotaxis (Zupo et al., 2016; Fink, 2007). In addition, evidences show that the "odour recognition" does not evolve according to feeding needs only (Jones and Flynn, 2005) but various species use chemical cues to interact with the surrounding environment, for example with the purpose of detecting the presence of possible predators, avoiding hazards (Maibam et al., 2014) or identifying suitable habitats (Fink, 2007; Watson and Ridal, 2004). Volatile organic compounds derived from the wounding of diatoms in the surroundings are important infochemicals, especially for vagile fauna associated to *P. oceanica* meadows (Jüttner et al., 2010).

If the role of diatoms and macroalgae in the production of volatile compounds, able to trigger specific reactions in aquatic invertebrate, has been recognized, this research represents the first attempt to explore how ocean acidification can influence the production of VOCs by algae and the recognition of those infochemicals by associated

invertebrates. Our results indicate that behavioural reactions may change according to the doses of VOCs offered, that different organisms react differently to algal VOCs, and that O.A. may tune the communication patterns and the relationships between algae and associated invertebrates in the *P. oceanica* communities.

As for the observed differences in the behavioural responses to various doses of infochemicals, they are quite common in this kind of studies on infochemicals. A bouquet of volatile compounds may sometimes trigger contrasting or even opposite responses according to the doses shown to the test organisms. Contemporarily, the reaction obtained by invertebrates in this study should be carefully interpreted due to the intrinsic difficulties to relate the concentration of VOCs in our tests with natural phenomena. We performed each test in static choice chamber (Zupo et al., 2015) and in flumes (Atema et al., 2002; Voigt and Atema, 1996) to inquire the relation between current speed and behavioural reactions, to check if current speed is an experimental variable and if it can interfere or modify our observations. In addition, contrasting responses, eventually produced by two different experimental systems, may be explained by the diverse interpretation of the same bouquet in two opposite experimental conditions: in the static chambers, tested organisms may perceive odour concentration gradients as a linear function that spreads homogeneously from the source until saturation of the experimental arena (Lof et al., 2007); in flumes, individuals perceive odours as pulse over the time (Voigt and Atema, 1996).

Results of our experiments indicate that the highest doses of VOCs produce repulsive reactions in the tested marine invertebrates. In *Alvania lineata*, *Enteromorpha prolifera* high doses (at both culture and test conditions) produced repulsive reactions. In *R. italiensis* the highest doses of *Enteromorpha prolifera* and *Diploneis* sp. VOCs (at normal conditions) produced a strong repulsion in both odour choice chambers. In adults of *H.*

inermis, on the contrary, the repulsion at high doses of VOCs was observed in flumes instead of a more complex response pattern observed in static choice chamber and these results were confirmed in juveniles of the same species where the repulsive pattern at the highest doses of VOCs was observed in flumes. In *Idotea balthica*, considering each alga at each condition, the highest doses of VOCs obtained the most repulsive scores in both odour choice chambers. The same pattern was observed in juvenile phase of this isopod, with negative scores obtained by high concentration of *Diploneis* sp. and *Enteromorpha prolifera* VOCs cultured and tested at normal condition. This complex pattern can be explained by a dose-dependent interpretation, by target organisms, of message carried out by VOCs. As aforementioned, in this case VOCs may bring a clear “danger” message, such as the presence of a predator or potentially dangerous grazers. The identification of a high concentration of VOCs is, indeed, compatible only with the presence of large-size organisms, such as fishes, that are wounding algae on the *P. oceanica* leaves. Thus, infochemicals that at low concentration carry an information about “the source of food”, at high concentration may be interpreted as a “danger: run-away” message.

As for the differences in the attractiveness/repulsion in different species, they should be linked to peculiar physiology and life strategies of each tested organisms. Thus, the same bouquet of odour may bring contrasting information to different target organisms according to their ecology and trophic role (Sbarbati and Osculati, 2006). This is the case, for example, of high concentration of *E. prolifera* VOCs (normal conditions) that produced a repulsion in adults of *H. inermis* and a strong attraction in adults of *I. balthica* or *C. scutellum parva* that produced an attraction in *A. lineata* and a repulsion in *R. italiensis*. The different localisation of gastropod species along the leaves of *Posidonia oceanica* may explain the opposing reactions observed. In fact, *Rissoa italianensis* lives on leaves in

shallow bed (1-3 m) which are heavily epiphytised by filamentous and encrusting macrophytes, while *Alvania lineata* lives at intermediate depths (3-10m) on leaves which are generally covered by diatoms (Gambi et al., 1992; Mazzella et al., 1992; Mazzella and Russo, 1989).

Therefore high concentration of VOCs may be interpreted as a different signal representing an attractant for some invertebrates that need to maximise the search for food, such as *I. balthica*, an invertebrate characterized by a high motile activity, and a repellent for other invertebrates (Fink, 2007), for which mimicry and defence represent the most important behavioural constraints (Lamberti et al., 1995), such as *H. inermis*. Indeed, since Hippolytidae are exposed to high predation pressure, their behaviour is influenced by the need to avoid predation (Bedini et al., 1997; Zupo and Nelson, 1997; Zupo, 1994) and consequently, the diffusion of large quantity of wound-activated VOCs may indicate the presence of predators chewing parts of *P. oceanica* and its epiphytes. *Idotea balthica*, living in low-energy *P. oceanica* detritus characterized by a high amount of poorly digestible structural carbohydrates (Sturaro et al., 2010), evolved a totally different interpretation of the odour, giving priority to the exploration of the area for a possible food source with respect to escape from possible predators. This species is probably accustomed to the odour of chewed algae and, as demonstrated for other species (Fink et al., 2006b), it is probably attracted by other animals grazing in the same area following an odour patch.

Our results indicate that the reactions are not only species-specific but, in addition, behavioural responses can change according to juvenile and adult stages. Indeed, differences in the attractive responses were observed between adults and juveniles in both *H. inermis* and in *I. balthica*. *Hippolyte inermis* adults were attracted by *Enteromorpha prolifera* at low (normal and acidified conditions) and medium

concentration (normal condition) and by *Diploneis* sp. (acidified condition). A reverse pattern of responses was observed in juveniles of the same species that showed an attraction for VOCs produced by *C. scutellum parva* at low concentration and medium concentration (normal conditions) as well as by *Diploneis* sp. at low concentration (normal conditions). Contrasting responses were observed in adults and juveniles of *I. balthica* too: adults were repelled by *C. scutellum parva* and attracted by *E. prolifera* and juveniles attracted by the diatom and repelled by the macroalga. It is well-known that larvae and juveniles of aquatic organisms use chemical cues to detect and reach the most favourable microhabitat (Ben-Tzvi et al., 2008, 2010; Pawlik, 1992; Herrnkind and Butler IV, 1986; Wetthey, 1986) and that selection of protected and preferred microhabitat is a key process to assure a successful recruitment. Our results indicate that juveniles of both species were attracted by diatoms bouquet, especially at low concentrations, instead of a repulsion observed in adults of the same species and that, between the two studied diatoms, *Cocconeis scutellum parva* produced the most contrasting results. Previous authors demonstrated that *H. inermis* (adult) is strongly repelled by VOCs produced by this diatom (Jüttner et al., 2010). We demonstrated, on the contrary, that juveniles are attracted by *Cocconeis* VOCs and we can hypothesize that this infochemicals are indicators of epiphytized *Posidonia oceanica* leaves. In fact, in a such a cryptic species, the search for “right” microhabitat may be crucial for their survival, playing a fundamental role as food source and shelter as well as to maintain the male/female ratio due to the apoptogenic effect of the *C. scutellum parva* secondary metabolite (Zupo et al., 2014; Nappo et al., 2012) on this species during the post-larval phase. The same behavioural response observed in juveniles of *I. balthica*, may be explained with the search for food sources too but not with the search for the correct microhabitat due to the fact that *I. balthica* individuals are hatched as fully functional juveniles (Fava et al., 1992; Tuomi et al., 1988;

Strong and Daborn, 1979; Strong, 1978), lacking a free larval phase. For this reason, fresh hatched larvae are born within the mother's environment, such as in a leaf debris, rich in shelters, where the species peculiar mimicry is fully functional and efficient (Camur-Elipek, 2009; Toonen et al., 2007).

Our results indicate that ocean acidification deeply affect VOCs production and recognition in *P. oceanica* meadows. Comparing the reactions produced by normal and acidified VOCs, contrasting results were observed in tested organisms. *Alvania lineata* and *Rissoa italiensis* produced an opposite pattern of responses if exposed to VOCs produced by normal and acidified *E. prolifera* and *Diploneis* sp., respectively. In *H. inermis* and *I. balthica* adults, ocean acidification had a relatively lower impact on the odour production and recognition with few behaviour differences observed.

Interesting differences were observed in response of *C. scutellum parva* VOCs produced and tested at acidified and normal conditions: in each tested organism, excepted for *H. inermis* adults, ocean acidification produced an alteration of behavioural responses. In both crustaceans, juveniles were attracted by VOCs produced and tested at normal pH, and with repulsion to the *C. scutellum parva* VOCs produced and tested at acidified conditions. *Cocconeis scutellum parva* is a benthic diatom that triggers specific physiologic reactions and induces the sex reversal in *H. inermis* (Zupo et al., 2007). The "wrong" reactions of *H. inermis*, exhibited in response to VOCs of *Cocconeis scutellum parva* cultured at acidified pH will produce a deep alteration of the coevolution of shrimp and diatom in *Posidonia* meadows.

In conclusion, we demonstrated that diatoms and macroalgae produce VOCs playing the role of infochemicals for invertebrates associated to the leaf stratum of *P. oceanica*. The conveyed information is dose-dependent, producing various responses according to VOCs concentrations. In addition, the responses are specie-specific, strictly related to the

ecological needs and life strategies of each species. Finally, our data suggest that increasing levels of CO₂ forecasted for the next decades could deeply modify the production and the recognition of infochemicals in animal communities associated to *P. oceanica*.

4.2 OCEAN ACIDIFICATION INFLUENCES PLANT-ANIMAL INTERACTIONS: A STUDY CASE ON APOPTOGENIC METABOLITES OF *COCCONEIS SCUTELLUM PARVA*.

4.2.1 ABSTRACT

Ocean acidification (O.A.) affects the ecology of oceans and it may produce interference in plant-animal interactions at various levels. Seagrass meadows located at acidified vents in the Bay of Naples (Italy) are considered as an open window to forecast the effects of global-changes on aquatic communities. *Cocconeis* is a genus of epiphytic diatoms, abundant in acidified seagrass meadows, playing a crucial ecological role in various marine environments. A still-unknown compound produced by *Cocconeis* demonstrated an apoptogenic power, triggering the suicide of the androgenic gland of *Hippolyte inermis* Leach 1915, a protandric hermaphrodite shrimp distributed in *P. oceanica* meadows as well in acidified vents. Feeding on *Cocconeis* sp. and intake of the diatom's apoptogenic compound was proven to be fundamental for maintaining *H. inermis* natural populations. Since O.A. deeply affects the physiology of diatoms, we wish to investigate how O.A. may influence the growth of *Cocconeis* and if, in future scenarios of O.A., *Cocconeis scutellum parva* will still produce its apoptogenic metabolites. We performed laboratory analyses to compare the growth of *Cocconeis scutellum parva* in *ad-hoc* designed photobioreactors, at two pH conditions (pH 7.7 and 8.2) and tested their apoptogenic power on *H. inermis* post-larvae. We demonstrated that the growth of *Cocconeis scutellum parva* is linearly correlated with the pCO₂ within the considered pH range and that diatoms cultured at acidified conditions changed their metabolite production. The production of apoptogenic compounds is reduced (or null) under the levels effective to trigger shrimp's sex reversal in O.A. conditions, and this will directly impact the stability of natural populations of *H. inermis*.

4.2.2 INTRODUCTION

Hippolyte inermis Leach is a shrimp mainly inhabiting meadows of *Posidonia oceanica* (Gambi et al., 1992) and in other seagrasses (Perez-Barros et al., 2004) and it is a key component of their food webs, as a link between primary producers, fishes and other carnivores (Zupo & Fresi, 1985). The shrimp naturally undergoes a process of protandric sex reversal (Yaldwyn, 1966; Veillet et al., 1963).

However, Zupo (1994) demonstrated the presence of two reproductive periods, the first in spring, developing both into males and females and the second in fall, with the appearance of males which shift to females (“alpha” females) during the next year. The presence of spring early-developed females (“beta” females) contributes to the fall reproductive burst and exhibits the maximum abundance in association with blooms of epiphytic diatoms (Zupo, 2000). The early-developed females demonstrated a peculiar dietetic pattern: in spring, gut contents of “beta” females are dominated by benthic diatoms and, among them, *Cocconeis* spp. are particularly abundant. In contrast, males and “alpha” females are generalist grazers feeding on common epiphytes of *P. oceanica* leaves such as micro and macroalgae, bryozoans and foraminiferids (Zupo and Fresi, 1985). It has been demonstrated that a) the ingestion of *Cocconeis* diatoms influences in a narrow time window the physiology of *H. inermis* (Zupo et al., 2008); b) the production of “beta females” is triggered by a still unknown lipophilic compound produced by diatoms after wounding and c) the compound has a highly selective apoptogenic power targeted on the shrimp’s androgenic glands (A.G.) (Maibam et al., 2014). The destruction of the A.G. in males of *H. inermis* by apoptosis represents a stabilizing factor for natural populations (Zupo, 2000) triggering an increase of ovigerous females during the fall reproductive season.

Various diatoms drastically change their productivity and growth dynamics, as well as the composition and concentration of secondary metabolites, in different culture conditions, influenced by light irradiance (Affan et al., 2007; Chundi et al., 2007), presence of pollutants (Eker-Develi et al., 2006), nutrient limitations (Göksan et al., 2003) and light spectrum (Raniello et al., 2007; Mouget et al., 2005).

In parallel, O.A. affects the ecology of oceans and the physiology of marine organisms and various direct and indirect effects on the marine biota are forecasted. Concentration of CO₂ in oceans increased in the last decades due to anthropogenic emissions (Diaz-Pulido et al., 2016) and a pH decrease in the order of 0.5 points is forecast for the next century (Turley et al., 2006; Raven et al., 2005). Thus, the chemistry of oceans and consequently various biological processes will be deeply affected by increasing concentrations of atmospheric carbon dioxide. O.A., combined with such factors as eutrophication and temperature rising, may cause a significant decrease in abundance and diversity of algae. Indeed, O.A. may have a deep effects on plant-animal interactions, algae growth, calcification rates of various algae as well as other physiological processes such as calcification (Bradassi et al., 2013; Gao and Zheng, 2010), nutrient uptake (Koch et al., 2013), and metabolisms (Porzio et al., 2013; Poore et al., 2013; Porzio et al., 2011; Hurd et al., 2009).

In addition, algae living in acidified environments may modify their patterns of production of secondary metabolites impacting marine food webs. In the “Castello” vents off the island of Ischia (Bay of Naples, Italy), considered as a natural laboratory to simulate future O.A. scenarios, Porzio et al., (2012) identified more than 22 diatom genera in the epiphytic community on *P. oceanica* leaves and *Cocconeis* was the dominant one. This study aimed at investigating changes in the production of the apoptogenic compound produced by *C. scutellum parva* when cultured at two pH levels, simulating the present

status and the forecast values for the year 2100. Since the shrimp is the only known “biological sensor”, able to track the presence of the active apoptogenic compound, *Cocconeis* diatoms were cultivated in the laboratory and tested on shrimp’s post-larvae, in order to detect any variation in the production of the compound due to the acidification. To compare the effects of *C. scutellum* var. *parva*, a strictly related variety, i.e., *C. scutellum* var. *posidoniae* was also tested, as a positive control.

4.2.3 MATERIALS AND METHODS

4.2.3.1 STOCK CULTURES AND INOCULATION PROCESS

Two *Cocconeis* varieties. were taken into consideration, *i.e.*, *C. scutellum parva* and *C. scutellum posidoniae*. Each species was cultured in continuous axenic conditions in 6 mL multi-wells containing 4 mL of Guillard's *f/2* medium with silica (Sigma-Aldrich, Milan, Italy). Cultures were kept under controlled conditions in a thermostatic chamber at 18 °C with 12:12 light:dark photoperiod. Light was provided by Sylvania GroLux (Osram Sylvania Inc., USA) at an irradiance of $140 \mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-2}$.

At the 16th day of grow-out, the surface of the multi-wells was almost completely covered by diatoms and cells were scraped and collected by a Pasteur pipette, then pooled in a sterilized beaker; the suspension was divided and transferred into 10 Petri dishes (diameter 7 cm) filled with 50 mL of *f/2* medium. At the end of the next grow-out phase (16 days), diatoms were collected by gently scraping off (with the aid of a Pasteur pipette) the bottom of culture vessels. Diatoms were pooled again in a sterilized beaker and the suspension was partitioned into three photobioreactors. Given the strong adhesive properties of *Cocconeis* spp., only part of diatoms inoculated in each photobioreactor could survive; for this reason, the diatom concentration in the suspension was not determined at the moment of the inoculation (Raniello et al., 2007).

4.2.3.2 PHOTOBIOREACTORS

Special photobioreactors adapted for benthic diatoms were *ad-hoc* designed to perform at normal and acidified conditions. Each photobioreactor was assembled using a Pyrex dish with a total volume of 2.4 L (300 mm x 200 mm x 40 mm; Fig. 4.1). Each reactor was constructed using a Pyrex dish (a) covered with a heat resistant glass plate (b). In the

opening at the center of the cover was housed a pH probe (InLab®Micro pH, Mettler Toledo; c). The pH probe was connected to a pH controller (SMS122, Milwaukee; d) that controls an electronic valve (e). The electronic valve is connected, on one side, to the CO₂ regulator (CO₂ Energy, Ferplast; f), and on the other side to a plastic stripette (g), fixed in a secondary opening in the glass cover. A centrifuge pump (Askoll Pure pump 300; h) was located on one side of the photobioreactor to avoid water stratification and was temporized by a microcontroller (EnerGenie EG-PMS2-LAN; i).

The vessel was covered with a heat resistant glass plate, provided with a narrow opening at its centre, where a pH probe was housed (InLab®Micro pH, Mettler Toledo). A secondary opening was placed sideways, where a plastic stripette was fixed. The InLab Micro probe is designed to work even in a reduced volume of water and up to a thickness of 3 mm. A pH controller (SMS122, Milwaukee) was connected to the Inlab Micro Probe (via a BNC cable) by an electronic valve connected in its turn to a CO₂ regulator (CO₂ Energy, Ferplast). To avoid water stratification and the formation of any pH gradient along the photobioreactor, a centrifuge pump (Askoll Pure pump 300) was added. The centrifuge pump was temporized by a micro-controller (EnerGenie EG-PMS2-LAN) that cyclically activated the pump each 30 minutes (1 minute on, 29 minutes off). The pH was regulated by a pH controller that opened and closed the electronic valve, when necessary, dispensing the CO₂ through a plastic stripette into the photobioreactors. The pH of the medium was checked five times a day to guarantee that pH oscillations were lower than 0.05.

Photobioreactors were used to culture *C. scutellum parva* at pH 7.7 and 8.2. In addition, *C. scutellum posidoniae* was cultivated at normal pH (8.2). Three replicates were produced for each diatom and condition. The grow-out of diatoms into photobioreactors continued for 16 days. To estimate the number of cells . mm⁻² into photobioreactors, 4

microscopy cover-slides (1 cm²) were placed into each photobioreactor. In each cover-slide, 24 areas of 0.004 mm² were randomly selected and examined under the inverted microscopy to record the number of cells grown. Each 3 days, 4 cover-slides per replica were examined, and the average number of cells present per surface area was computed. The average number of cells for each species of diatoms and standard deviations among replicates were also computed. Diatom transfers and collections were performed under a laminar flow hood and all dishes and culture instruments were previously sterilized at 120 °C. All the intact cells of benthic diatoms strongly adhere to the bottom of glass cups 24 hours after the inoculation.

After 16 days the medium in each photobioreactor was removed and the vessels were quickly rinsed with distilled water to remove residual salts. Emptied vessels containing a diatom film on their bottom were immediately frozen at -20 °C, then freeze-dried. Dry diatoms were scraped off using an iron blade, then weighed and kept in dry vessels at -20 °C up to their use for bioassays on shrimps.

4.2.3.3 BIOASSAYS ON *HIPPOLYTE INERMIS*

We followed the techniques described by Zupo and Messina (2007) to test the effect of diatom's secondary metabolites on the target shrimp *Hippolyte inermis*. Ovigerous females of *H. inermis* were collected in a *Posidonia oceanica* meadow off Castello Aragonese (Island of Ischia, bay of Naples, Italy), sorted on boat and kept in plastic bags to be transferred to the laboratory. Ovigerous females were then transferred into 2 L conical flasks (2 ind. in each flask), containing 1.8 L of filtered seawater and small portions of *P. oceanica* leaves were added to provide a shelter for shrimps. They were kept at 18 °C in a thermostatic chamber at a mean irradiance of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a photoperiod of

10:14 h light : dark. Females releasing a variable number of larvae (20-400) in the following 10 days were returned to sea.

Larvae produced were collected daily and transferred to 1 L conical flasks containing 800 mL of filtered seawater in pools of 80 ind. Larvae were fed with *Artemia salina* nauplii and *Brachionus plicatilis* (4 individuals per mL) for 7 days. *Artemia salina* nauplii were enriched daily with Algamac BioMarine (Hawthorne, CA, USA). Survival rates were recorded daily, collecting larvae by a Pasteur pipette and transferring them into a fresh culture medium. Larvae already metamorphosed into post-larvae (in about 26 days) were randomly pooled and further divided into groups of 25 post-larvae to be individually transferred into crystallizing dishes (14 cm diameters) containing 400 mL of filtered seawater. Negative controls were fed a base feed composed of equal proportions of SHG “Artemia Enriched”, SHG “Microperle” and SHG “Pure Spirulina” (produced by Super High Group, Ovada, Italy). Treatments were fed on a basic food containing dried cells of *C. scutellum parva* cultured at pH 7.7 or 8.2, added to in a ratio of 2:1 (w/w), according to treatments. Positive control replicates were fed on a base food added with dried cells of *C. scutellum posidoniae* at the same proportions used for the bioassays previously described. Dry feeds were prepared and stored at -20 °C. Each post-larval replicate received daily a 5 mg ration of feed. Post-larvae medium was daily replaced, the crystallizing dishes were washed and shrimps transferred. Post-larvae aged 40 days were sacrificed and fixed in 70% ethanol. Their total body length was measured using millimetric paper under a dissecting microscope (Leica Z16 APO) and pleopods II were cut, mounted on a slide and observed under an optical microscopy (Leica DMLB) to determine their sex based on the presence/absence of a masculine appendix (Mutalipassi, Maibam, et al., 2018).

4.2.3.4 STATISTICAL ANALYSES

Average survival rates in larval cultures were evaluated and plotted by Prism 7 (Graph-Pad Software, La Jolla, USA). Diatom growth curves were computed according to the equation:

$$Y = (Y_0 - a) \cdot e^{(-b \cdot X)} + a$$

where:

“a” is the Y value at infinite times;

“Y₀” is Y value when X is zero;

“b” is the rate constant, expressed in reciprocal of the X axis time units;

The growth curves obtained for *C. scutellum parva* at the two pH conditions was compared by a paired t-test.

Analyses of the pCO₂ were performing using the CO₂Sys EXCEL Macro proposed by Pierrot et al., (2006) of Carbon Dioxide Information Analysis Center (Oak Ridge National Laboratory, U.S. Department of Energy, Tennessee). Growth rates were normalized to pCO₂ concentration at the two growing conditions according to the equation:

$$\text{Normalized growth rate} = (\text{Number of cells} \cdot \text{mm}^{-2}) \cdot \text{pCO}_{2-1}$$

A Spearman correlation analysis of normalised growths at the two different pH conditions was performed using Prism. The percentage of females normalized to the total number of mature individuals (% Female . mature individuals⁻¹) was computed. The % female . mature individuals⁻¹ (F/mat) index permits to determine the apoptogenic effects of diatom compounds on mature *H. inermis*, avoiding the bias due to immature individuals or shrimp with corroded pleopods due to bacterial infections. The significance of

differences among treatments and controls was tested by one-way analysis of variance (ANOVA), adopting the Tukey's multiple comparisons post-hoc test (Prism software) to the observed F/mat scores.

4.2.4 RESULTS

4.2.4.1 PHOTOBIOREACTORS AND DIATOM CULTURES

Photobioreactors here developed permitted to keep the pH of the medium constant during the whole experimental period, by adjusting the pCO₂ with small additions of gas immediately dissolved by the movements of the applied pumps. The maximum deviation of the pH from the values set on the control instrument was 0.05. At the end of the culture periods, we observed an average pH of 7.695 (± 0.033), 8.19 (± 0.046) and 8.2 (± 0.030) in *C. scutellum parva* cultures at pH 7.7, at pH 8.2 and in *C. scutellum posidoniae* cultures, respectively.

Diatom growth curves recorded at pH 7.7 (pCO₂ 1304.2 μ Atm) and 8.2 (pCO₂ 342.8 μ Atm) are quite different at the steady phase, starting from similar densities (Fig. 4.8). In fact, the cell density at the beginning of the experiment (24 hours after inoculation) in replicates at pH 7.7 and 8.2 was 14183 (± 3975) and 3546 (± 994) cells . mm⁻² , respectively.

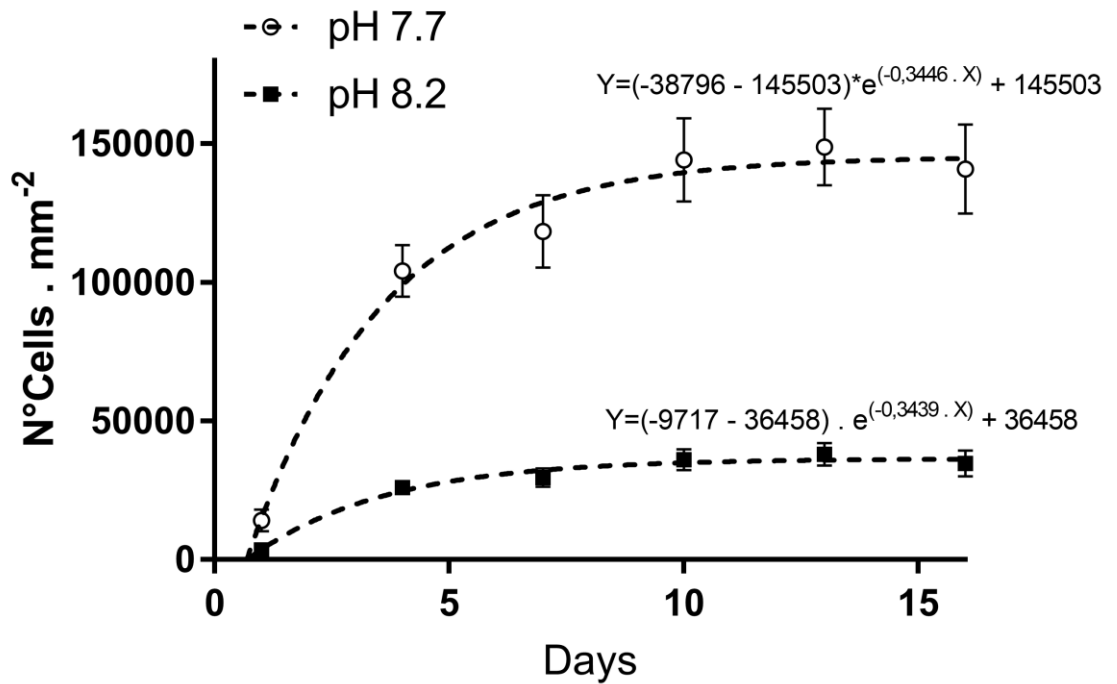


Fig. 4.8: Growth curve of *Cocconeis scutellum parva* at pH 7.7 (white dots) and at pH 8.2 (black square) obtained over 16 days of culture. Error bars indicate differences among cell counts obtained in the same day in all replicates.

Growth saturation and steady state were consistently reached within 10 days of incubation at both tested conditions (Fig. 4.8). *Cocconeis scutellum parva*, cultured at two pH conditions (pH 7.7 and 8.2) produced significant differences in the growth rate (t test; $P \leq 0.01$). The highest growth rates were recorded at pH 7.7, with 144283 (± 15048) and 148808 (± 13935) cells . mm⁻² reached at the days 10 and 13, respectively. *Cocconeis scutellum parva* cultured at pH 8.2 produced, at the same times, concentrations of 36,070 ($\pm 3,762$) and 38,066 ($\pm 4,166$) cells . mm⁻², respectively. A significant correlation was demonstrated between the normalised growths at the two different pH conditions (Spearman test, $P \leq 0.01$, $r_s = 1$).

4.2.4.2 LARVAL AND POST-LARVAL PRODUCTION

On average 81.8 (± 19.3) larvae were produced by each of ten ovigerous females. During 24.5 (± 1.08) days of larval growth, 78.26 % (± 3.6) of survival was recorded (Fig. 4.9).

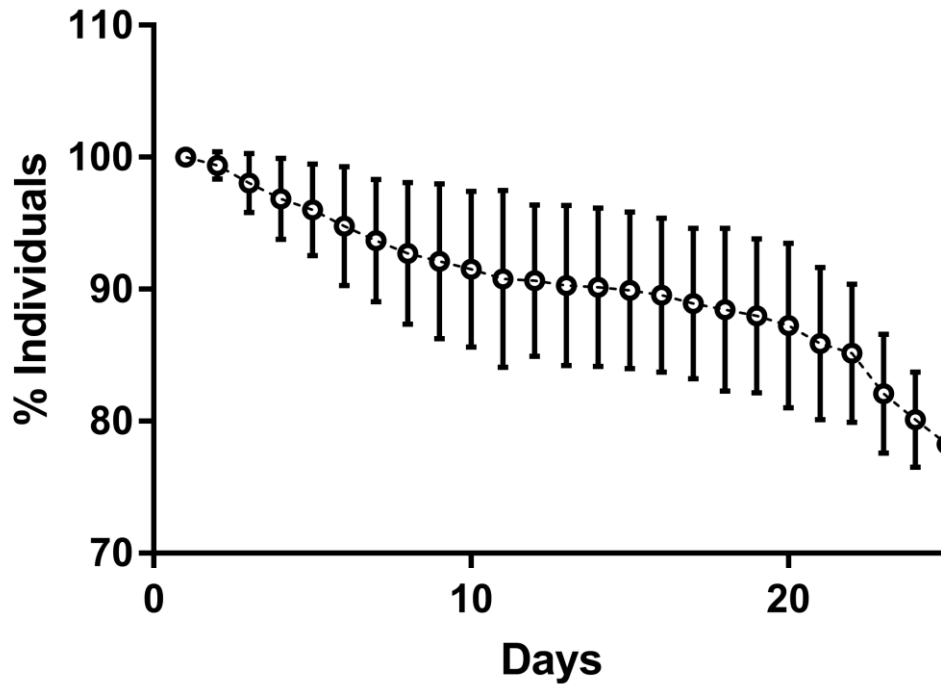


Fig. 4.9: Percent survival rates of *Hippolyte inermis* cultured 26 days in glass conical flask (dots) and their standard deviation (vertical bar).

After 25 days of larval culture all the individuals settled and metamorphosed into post-larvae. A survival rate of 80 (± 4.9) %, a percentage of 6.4 (± 4.6) % immature individuals and an average size of 7.63 (± 0.65) mm were recorded in fixed shrimps, at the end of post-larval culture. Treatments fed on *C. scutellum parva* cultured in acidified conditions (pH 7.7) produced a survival of 87.2 (± 7.7) %, a percentage of immatures of 7.2 (± 5.9) % and an average size of 7.52 (± 0.63) mm. In treatments fed on *C. scutellum parva* cultured

at normal conditions (pH 8.2) a survival of 82.4 (± 2.2) %, 4.0 (± 4.0) % of immatures and an average size of 7.80 (± 0.60) mm were recorded.

4.2.4.3 BIOASSAY

Treatments fed on *Cocconeis scutellum posidoniae* produced a survival of 86.4 (± 5.4) %, a percentage of immature of 4.8 (± 1.8) % and an average size of 7.75 (± 0.63) mm. All individuals were subjected, during the post-larval phase, to the same culture conditions and they were cultured contemporaneously in the same thermostatic chamber. The highest activity (highest ratio of sex reversed individuals, evaluated according to the presence/absence of *masculinae* appendices on shrimp's pleopods) was measured in replicates fed on *C. scutellum parva* cultured at normal conditions (pH 8.2), where we observed 68.5 (± 2.8) % female . mature individuals⁻¹ (Fig. 4.10).

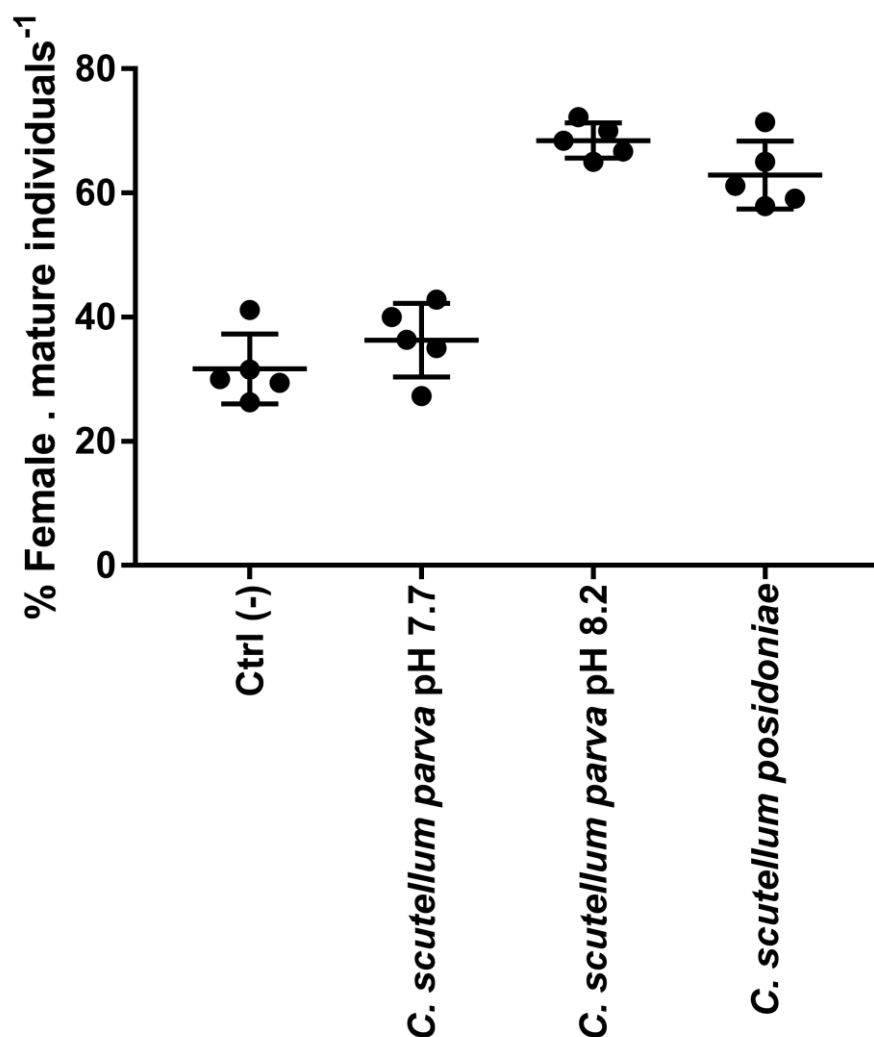


Fig. 4.10: Female / mature ratios obtained for each diet. Average value, standard deviations and the value of each replicate are reported.

Positive controls exhibited apoptogenic activity on shrimps, with 63.4 F/mat (± 2.8) % in replicates fed on *C. scutellum posidoniae*. Replicates fed on *C. scutellum parva* cultured in acidified conditions (pH 7.7) as well as negative controls, produced scarce or null activities on *H. inermis* post-larvae triggering 36.3 F/mat (± 5.9) % and 31.7 F/mat (± 5.6) %.

Significant differences among treatments were indicated by ANOVA ($P \leq 0.0001$). Notably, no differences in the percentage of F/mat were observed between negative controls and *C. scutellum parva* cultured at acidified conditions (Tukey's, $P \geq 0.05$) as well as between *C. scutellum parva* cultured at normal conditions and *C. scutellum posidoniae* (Tukey's, $P \geq$

0.05). In contrast, significant differences were found between *C. scutellum parva* cultured both at normal and acidified conditions and negative controls (Tukey's, $P \leq 0,0001$).

4.2.5 DISCUSSION

It has been demonstrated that *Posidonia oceanica* meadows growing in acidified conditions show altered epiphyte and vagile fauna communities (Hall-Spencer et al., 2008), with a strong reduction in organisms bearing aragonite skeletons. Our results on the growth of *C. scutellum parva* in photobioreactors are in accordance with studies performed at Castello Aragonese meadows, where species associated to the vent environments include a suite of organisms resilient to naturally high concentrations of pCO₂ and the massive presence of *Cocconeis* spp. may indicate that these species may be strongly advantaged by this pH condition, in the field. The effect of CO₂ on the growth of diatoms is quite complex and, probably, depends on the peculiar physiology of each species. Elevated CO₂ concentration did not cause significant differences in growth in diatoms such as *Asterionella glacialis*, *Thalassiosira punctigera*, *Coscinodiscus wailesii*, *Phaeodactylum tricornutum* (James et al., 2014; Li et al., 2012; Burkhardt et al., 1999). In contrast, Wu, Campbell, Irwin, Suggett, & Finkel (2014) showed an advantage of larger planktonic diatom species, more than 40 µm in diameter, over smaller-sized ones with an enhanced growth rate under elevated pCO₂ due to a combination of increased diffusion rates, a lowering of metabolic costs and a lower susceptibility to photo-inactivation of PSII.

A completely different situation was experienced in our experiments, where *Cocconeis scutellum parva* cultured at acidified conditions (pH 7.7) produced four times more cells than the same diatoms cultured at normal conditions (8.2). The difference in growth was directly correlated to the pCO₂ into the cultures. In fact, normalising the data to the pCO₂ calculated at two different pH, we observed no significant differences between treatments.

In order to culture these highly adhesive benthic diatoms, the use of photobioreactors is convenient due to low operational time and perfect repeatability of procedures and it permits an optimization of space in thermostatic chambers (Raniello et al., 2007) avoiding time consuming procedures of cultures in Petri dishes (Zupo et al., 2014). Various custom-made photobioreactors were designed to mass culture planktonic (Beuzenberg et al., 2017; Ozkan and Rorrer, 2017; Yen et al., 2014) and low adhesive benthic microalgae (Silva-Aciares and Riquelme, 2008; Avendaño-Herrera and Riquelme, 2007; Lebeau et al., 2002) but a few of them are specific for high adhesive benthic diatoms and, at the same time, are capable of manipulating pH (Granum, 2002). The photobioreactor here described was proven to be effective in manipulating the pH in microalgal cultures and, in addition, they demonstrated to be capable of culture slow-growing, highly adhesive, benthic diatoms in axenic conditions.

Cocconeis sp., particularly abundant in the field at both normal and acidified areas of Castello Aragonese (Ischia, Naples, Italy), are a food source of *H. inermis* as demonstrated by the abundance of their thecae in its gut contents (Zupo, 2001), especially in spring. Diatoms have been demonstrated to influence the ecology and the life cycle of other crustaceans (Barreiro et al., 2011; Miralto et al., 2003; Buttino et al., 1999) but in this species, according to co-evolutionary processes (triggering, in the shrimp, the development of beta females due to apoptotic disruption of the male gonadic buds; Zupo, 2001), the toxic effect of diatoms are translated into spring signals to synchronise the reproductive cycle to the abundance of epiphytes (Zupo, 1994). Although it is known that acidification produces a change in the set of secondary metabolites produced by diatoms (Poore et al., 2013), here we demonstrated a strong reduction in the production of the apoptogenic compound produced by *Cocconeis* sp. at pH 7.7. Although *H. inermis* is a polytrophic species (Zupo, 2001), it strongly depends on *Cocconeis* sp. to keep the size of

natural stocks constant. The development of beta females has been demonstrated to be a crucial factor in maintaining a constant sex ratio in this species, allowing for a fall large reproductive burst (Zupo, 1994).

To obtain correct bioassays responses, the quality of shrimp larvae is of primary importance. For this reason, it is important to follow survival and growth, in larval and post-larval cultures of *H. inermis*, to evaluate their specific stress levels (Calado et al., 2009; Racotta et al., 2003). The reduction of stress factors, in studies on physiology of model organisms, should be taken into account to avoid bias in the reaching of actual sex ratios. Indeed, it was demonstrated that stress may influence sex ratios in protandric decapods (Zupo and Messina, 2007; Calado et al., 2005; Bauer, 2000). Number of larvae produced by each female (81.8 ± 19.3), low larval mortality and the duration of the larval period were positively related to the health status of cultures in previous studies on *H. inermis* (Mutalipassi, Di Natale, et al., 2018; Zupo et al., 2008; Michèl, 2007; Zupo and Buttino, 2001; Zupo, 2000) and they may indicate absence of stress in cultured shrimps. A further demonstration of the low level of stress reached in larval and post-larval cultures was given by the low mortality, the size reached by most shrimps at the end of the feeding experiments and the low female/mature ration observed in negative controls, as compared to previous studies (Zupo et al., 2008; Zupo and Messina, 2007; Zupo, 2000).

Our bioassays confirmed the activity of the apoptogenic compounds produced by diatoms representing our positive control (*C. scutellum posidoniae*), targeted on the androgenic gland of *H. inermis* post-larvae (Zupo et al., 2008; Zupo and Messina, 2007; Zupo, 1994, 2000, 2001). It is worth observing that *Cocconeis scutellum parva* cultured in normal conditions (pH 8.2) was the most effective diatom with a $68.5 (\pm 2.8)$ % females. mature individuals⁻¹. On the contrary, *Cocconeis scutellum parva* cultured in acidified conditions

produced the lowest female/matures ratio, with no significant differences in comparison to negative controls.

The correlation of the life cycle of *H. inermis* with the seasonal patterns of abundance of epiphytic *Cocconeis* sp. in *Posidonia oceanica* meadows (Zupo, 1994) will be altered by climate changes. In the future, *H. inermis* might be still able to find *Cocconeis* sp. in the field, as the dominant epiphyte in spring acidified meadows (Garrard et al., 2014), but the plant-animal co-evolutionary relationship will be probably lost, due to changes in the secondary metabolites produced by the microalga. For this reason, *Hippolyte inermis* could miss, in future, the possibility to obtain crucial infochemicals (e.g., the still unidentified apoptogenic compound) fundamental for triggering the apoptosis of cells in the androgenic gland of these shrimps facilitating its sex reversal. Thus, an increase of the availability of its diatom food due to a faster turnover of microalgae, will correspond to a lack of key metabolites in an acidified environment, indispensable to assure its smart life strategy.

The present study demonstrates that, besides basic processes directly influencing the life and the production of marine organisms, more complex mechanisms will determine the future of marine associations in acidified oceans.

5 : CONCLUSIONS

This Ph.D. project aimed at reaching realistic methods to choose a model organism to investigate specific scientific issues. We approached this scientific question by integrating data obtained by previous researches and new tests specifically performed on a selected group of organisms, from single-celled micro-models, such as Cyanobacteria, to macro-models, such as Echinoderms, the invertebrate phyla phylogenetically close to chordates (Swalla and Smith, 2008; Jefferies et al., 1996; Turbeville et al., 1994). The topic of this Ph.D. project is broad and it can be approached from various points of view, virtually embracing all fields of experimental scientific research. Due to the large use of models in all fields of research, it was impossible to evaluate during the 3 years of project all aspects related to this topic. For these reasons, we decided to focalize the attention to the ecotoxicology, to apoptosis and to chemical ecology and infochemical fields, which can be considered as examples of many key areas of the scientific research.

To perform our experiments, we used model species belonging to various taxa, especially those associated to seagrass complex ecosystems. Unicellular models are microalgae living as epiphytes on *P. oceanica* leaves: the diatom *Cocconeis scutellum* and the cyanobacterium *Halomicronema metazoicum*. They were both used as sources of interesting molecules to be tested on animal models. At the same time, we performed some tests on those two species in order to understand their physiology and optimize the culture techniques. As target organisms we used two standardized small metazoans, such as *Brachionus plicatilis* and *Artemia salina*, and three macro organisms, such as *Hippolyte inermis*, *Idotea balthica* and *Paracentrotus lividus*.

We assigned a score to each considered parameter, in order to compile a two-entrance table, according to the following rules:

Size	1	Organism size less than 0.1 mm
	2	Organism size between 0.1 mm and 1 mm
	3	Organism size higher than 1 mm
Management	1	Maximal difficulty
	2	Intermediate difficulty
	3	Minimal difficulty
Reproduction in laboratory	1	Seasonal reproduction - hard to breed
	2	Intermediate parameters
	3	Continuous reproduction - easy to breed
Rearing protocols set	1	Protocols and Systems not yet available
	2	Adaptable protocols and culture systems available
	3	Specifically designed protocols and culture systems available
Easy availability	1	Hard to obtain the species from field or commercial market
	2	Species are rare or not easily available in field or in commercial market
	3	Easy to obtain the species from field or commercial market
Stress responses	1	Weak or absent responses to stressors
	2	Intermediate responses to stressors
	3	Many responses to stressors
Apoptosis	1	No data or no response available
	2	Weak responses or production of apoptogenic compounds
	3	Strong responses or production of apoptogenic compounds
Chemical ecology and infochemicals	1	No data or no response available
	2	Weak responses or production of infochemicals and secondary metabolites
	3	Strong responses or production of infochemicals and secondary metabolites
Molecular biology	1	No molecular protocols and/or genes available
	2	Few protocols, some genes available
	3	Protocols available, many genes or metabolomics data
Genome / Transcriptome	0	No genome available for the selected species or phylogenetically related ones
	1	Genomes or transcriptomes of related species available
	2	Genome and/or transcriptome annotated

Table 5.1: Criteria of score assignment for considered field.

The application of the above reported rules (Table 5.1) permits to produce a general ranking of some key m.o. (Table 5.2) by characterising each of them according to the results of this thesis integrated with literature data, as specified in the following sections.

		<i>Cocconeis scutellum</i>	<i>Halomicronema metazoicum</i>	<i>Brachionus plicatilis</i>	<i>Artemia salina</i>	<i>Hippolyte inermis</i>	<i>Idotea balthica</i>	<i>Paracentrotus lividus</i>
Cultures	Size	1	1	2	2	3	3	3
	Management	1	3	3	3	1	3	2
	Reproduction	3	3	3	3	1	3	2
	Rearing protocols set	3	2	3	3	2	1	2
	Easy availability	1	2	3	3	2	3	3
Application fields	Stress responses	2	2	3	1	2	1	3
	Apoptosis	3	2	1	2	3	1	3
	Chemical ecology and infochemicals	3	3	3	1	2	2	1
Other features	Molecular biology	1	2	2	3	1	1	3
	Genome / Transcriptome	0	1	1	2	0	2	1

Table 5.2: Model organisms ranking table, compiled according to four arbitrary scores that were chosen based on the research herein reported, integrated with literature data.

5.1 MICRO-MODEL ORGANISMS

Cocconeis scutellum parva is a species broadly used in scientific researches due to its size and morphology (Size score: 1), allowing for an easy identification in light microscopy (De Stefano et al., 2008; Romero, 1996; Romero and Rivera, 1996). It is one of the most commonly reported benthic species in ecological (Buric et al., 2004; Borum et al., 1984; Jacobs and Noten, 1980) and taxonomic studies, with a large variety of studies that focus solely on this species and its intraspecific taxa (De Stefano et al., 2000, 2008; Gaul et al., 1993). In addition, this species and the related genus are used to study changes in diatom microfossil assemblages (Vos and De Wolf, 1993) associated with marine catastrophic events such as tsunami and earthquakes (Dawson, 2007; Chagué-Goff et al., 2002; Dawson et al., 1996), and to record relative sea-level history and environmental changes (Sato et al., 2016; Romundset et al., 2010; Jiang et al., 1997). It is typical of low light regimes and shaded environments and it is characterized by a slow growth at laboratory conditions. Our studies demonstrated that *C. scutellum* is quite hard to culture despite its reproductive and developmental features are well studied (Mizuno, 1987; Mizuno and Okuda, 1985). In addition, although it is possible to inoculate and reproduce it through the year at laboratory conditions (Reproduction score: 3), intrinsic difficulties of the isolation in field, the slow growth as well as the high adhesiveness and the fragility of its thecae are big disadvantages of this species complicating the culture and the harvest activities (Management score: 1; Easy availability score: 1). This is particularly true if we compare *C. scutellum parva* to other diatom models, such as *Pseudo-nitzschia*, *Phaeodactylum* or *Talassosira* species (Hennon et al., 2015; Sabatino et al., 2015; Taucher et al., 2015; Alverson et al., 2011; Thessen et al., 2009; Sumper and Brunner, 2008; Cutignano et al., 2006; Lopez et al., 2005; Fehling et al., 2004). In addition, slow growth diatoms are more sensible to be contaminated by saprophytic bacteria, protozoans as

well as other, faster, diatoms (Thurmond and Kroth, 2007; Management score: 1). As demonstrated in the chapter n° 4, paragraphs 4.2.4 and 4.2.5, the growth rates may be improved by the use of CO₂ as a fertilizer but at the cost of a change in diatom metabolism. Nevertheless, we demonstrated that photobioreactors designed to culture benthic diatoms simplify the culture and the management of highly adhesive diatoms (Rearing protocols set score: 3) as demonstrate by other authors too (Raniello et al., 2007). The ability to adapt to a high CO₂ world, as demonstrated by this thesis and by field studies (Porzio et al., 2012), make this species a good candidate to study adaptations and responses of diatom to climate changes although few studies are available (Stress responses: 2). In both experiments reported in the “Plant animal interaction” chapter, a change in the production of secondary metabolites (the apoptogenic compounds) and of infochemicals if the diatom is cultured in acidified conditions was recorded. For these reasons, it has been proven to be not only a good model to study metabolic alteration but also an interesting source of infochemicals in studies in plant-animal interaction studies. In fact, we demonstrated that, in acidified conditions, the communication between this species and the vagile fauna sharing the same environment is largely altered (Chemical ecology and infochemicals score: 3). Finally, *C. scutellum parva* is studied due to its importance as a source of apoptogenic compounds (Zupo et al., 2014; Nappo et al., 2012) and due to its fundamental role on as food for grazers on the *P. oceanica* leaves (Zupo and Maibam, 2011; Jüttner et al., 2010; De Stefano et al., 2008; Kawamura et al., 2004; Takami et al., 1997). The co-evolutive relationship *Hippolyte-Cocconeis* can lead to the development of new molecules with pharmacological activity and to a deeper understanding of the cellular mechanisms of apoptosis (Apoptosis score: 3).

As a study organism, *C. scutellum parva* has been proven to be a complex model to be approached with molecular tools. Up to date, its genome and transcriptome are not

available (Genome / Transcriptome points: 0) and difficulties in the extraction of genetic materials have been encountered by our partner workgroups. The failing of DNA extraction could be due to some major constraints in the harvest and in the culture method and not be related to specific genomic characteristics of this diatom. In fact: a) Cultures of *C. scutellum parva* were not totally axenic and, using the 16S sequencing, we obtained a high pairwise sequence identity with bacteria and non-diatom algae; b) during the harvesting process, which involves the use of a blade scraper, a large quantity of diatoms broke (due to high adhesive power characterizing this species) with the irremediable loss of genetic materials in the culture medium (Molecular biology score: 1).

Differently from *C. scutellum*, the cyanobacterium *Halomicronema metazoicum* has been recently isolated and only a few data about its physiology, biology and ecology are available. It is a mats forming cyanobacterium with cylindrical and elongated cells (size of 2–5 μm ; Size score: 1). We do not have data about the distribution of the species in nature but, considering our observations, it seems quite abundant on *P. oceanica* meadows around Ischia island (Italy; Easy availability score: 2). We tested the growth of this species in the laboratory using a standard *f/2* medium (*f/2* with silicates, Sigma Aldrich) at three salinities, light conditions and temperatures. Nevertheless, we didn't use specifically designed photo-bioreactors, and despite the fact, *H. metazoicum* demonstrated to be an organism easy to culture (Rearing protocols set score: 2; Reproduction score: 3). Indeed, it is well adaptable to laboratory rearing conditions without the need of highly specialized personnel or specific culture medium and that can be inoculated continuously merely cutting the cyanobacteria mats (Management score: 3). In addition, it has been demonstrated to be an easy to culture organism able to produce various bioactive compounds. These compounds exhibited both antimutagenic and cytotoxic activities. Despite the fact we don't actually know if the bioactive compounds are

secondary metabolites, bioactive metabolites released in the spent medium exhibit an allelopathic activity with detrimental effects on various organisms tested (Chemical ecology and infochemicals score: 3).

Halomicronema metazoicum has been used as a source of active compounds and we tested its activity through bioassays on a range of target organisms: *Artemia salina*, *Brachionus plicatilis* and *Paracentrotus lividus* embryos. In addition to our tests, that demonstrated a cytotoxic and antimitotic potential, *Halomicronema* species, as well as the phylogenetically strictly related *Phormidium* species, have been proven to produce photosynthetic macromolecular protein complexes. Among the produced compounds, C-phycoerythrin was identified as an interesting apoptogenic compound triggering apoptosis and cell arrest (at G0/G1 phase) against A549 human lung carcinoma cells (Apoptosis score: 2). Further investigations will aim at discovering active molecules in our microorganism's strain, both new, unknown, molecules as well as modifications of already known molecules, also showing apoptogenic power. To date we do not have any information about adaptations of this cyanobacterium to global changes and it is impossible to give an objective score on this topic, although the differences in the secondary metabolites production observed in cyanobacteria cultured at various culture conditions can be considered as a stress response (Stress responses score: 2). Due to the recently isolation of this species no transcriptomes or genome are available to date but, the genome of the congeneric species *Halomicronema hongdechloris* (https://www.ncbi.nlm.nih.gov/nuccore/NZ_CP021983.1) is available as well as for the phylogenetically related *Phormidium* sp. (Nelson et al., 2016; Genome / Transcriptome score: 1). In addition, molecular tools are available for *Halomicronema* and *Phormidium* (Katarzyna et al., 2011; Strunecký et al., 2010; Comte et al., 2007; Marquardt and

Palinska, 2007; Pfeiffer and Palińska, 2002) and 16S sequencing was performed using a published protocols (Ruocco et al., in press; Molecular biology score: 2).

5.2 METAZOAN SMALL MODELS

Among metazoan model organisms, rotifers are small (Size score: 2), simple in their tissue organization and easy to cultivate. In addition, rotifer's populations are genetically homozygous, providing clones for laboratory tests (Dahms et al., 2011; Hagiwara et al., 2007; Hagiwara, 1994). They are generally harmless for aquatic organisms and humans, with the exception of some species that can play the role of true parasites (May, 1989). They are widely distributed and ecologically important in freshwater environments due to their role of basal consumers mainly feeding on other microscopic organisms, including bacteria, algae and protists (Wallace and Smith, 2013). Among the various species of rotifers, we used in our tests *Brachionus plicatilis*, a rotifer commonly used in aquaculture (Das et al., 2012; Lubzens et al., 1989; Easy availability score: 3). This model has some advantages and its culture methods are highly standardized (Hagiwara et al., 2007, 2017; Kostopoulou et al., 2012; Yoshimura et al., 2003; Hagiwara, 1994; rearing protocols set score: 3). In addition, it has some unique features that make it a good model organism, such as high turnover rates, high population density, rapid population growth rates and parthenogenetic reproduction as well as the possibility to store resting eggs for a longer period of time (Snell, 1992; Fu et al., 1991; Theilacker and McMaster, 1971; management score: 3). Its reproductive physiology is well studied with a continuous reproduction through the year at laboratory conditions (Reproduction score: 3), although other rotifers were preferred as models for developmental biology studies (Ricci and Boschetti, 2003), *Brachionus plicatilis* has some interesting features, such as the eutely and a transparent body (Wallace, 2002; Nogrady et al., 1993).

Thanks to these features, *Brachionus plicatilis* became a useful model system for studies in aquatic ecology (Starkweather, 1980; Hino and Hirano, 1976), in population dynamics (Snell et al., 2006; Yúfera and Navarro, 1995; Stemberger and Gilbert, 1985) as well as in speciation (Gómez et al., 2002) and evolutionary ecology (Stelzer et al., 2011; Campillo et al., 2011; Suatoni et al., 2006a, 2006b; Gómez et al., 2002; Serra et al., 1998). On the contrary, a few papers concerning apoptosis are available for this species up to date (Apoptosis score: 1).

It has a long story as model organism in toxicological investigation (Sha et al., 2015; Garaventa et al., 2010; Marcial et al., 2005; Snell and Persoone, 1989) and we tested Cyanobacteria spent medium, at different concentrations, on adult individuals. Despite the lower sensitivity showed by *B. plicatilis* treated with *H. metazoicum* medium, if compared to *P. lividus* larvae, it is impossible to state if the cytotoxic activity observed in rotifers bioassays is due the same active molecule acting as antimitotic on the sea urchin. Undoubtedly, in the cyanobacterium spent medium there was a complicate mix of different active compounds that act at different level and with a various effect on metazoans physiology. Due to this hypothesis, we cannot state that *B. plicatilis* has a lower sensibility to active compound if compared to *P. lividus* larvae (Chapter: 3, paragraphs 3.2.4 and 3.2.5). This hypothesis is supported by results obtained from *Artemia salina* bioassay showing a not dissimilar cytotoxicity at the same spent medium dilutions (Chapter: 3, paragraphs 3.1.4 and 3.1.5). We can confirm here that *B. plicatilis* is a good model to detect cytotoxic compounds.

Kostopoulou and Centre (2012) determined the potential of *B. plicatilis* in climate changes studies due to its unique and desirable characteristics: a) temperature is the most important factor shaping the population dynamics of rotifers (Gaudy et al., 1995; Miracle and Serra, 1989; Arndt, 1988; Galkovskaja, 1987) and it is expected that temperature

variations will affect *B. plicatilis* natural populations; b) no data are available about tolerance of *B. plicatilis* to low pH but this parameters can be easily tested experimentally using indices such as swimming speed, respiration and filtering rate (Locke, 1991; Epp and Winston, 1978); c) climate changes affect natural population of rotifers. Using long-term studies on the distribution of *B. plicatilis* it can be possible to discern the influence of climate changes on this species by comparing past distributions with the present ones; sexual reproduction in rotifers is a more sensitive processes to externa influences (Serra et al., 2004; Snell and Carmona, 1995; Snell and Boyer, 1988), in part due to its reliance on chemical communication (Snell et al., 2006) that, we demonstrated on other models, it is deeply affected by water acidification (Stress response score: 3).

Brachionus plicatilis, and some closely related species, although not extensively studied, has been used to study plant-animal interactions by investigating a) alterations of feeding rates induced by chemical cues (Verschoor et al., 2007), b) herbivore-induced defences (Van Der Stap et al., 2006), c) morphological (Green and Lan, 1974) and behavioural (García et al., 2007) changes induced by the presence of predator kairomones as well as the effect of infochemicals produced by *B. plicatilis* (Yang et al., 2008) on other organisms (Chemical ecology and infochemicals score: 3).

Although molecular tools and protocols were available, genetic resources available to the rotifer community have been restricted to few genes and some other genomic markers like telomeric regions (Gladyshev and Arkhipova, 2007), transposons (Arkhipova and Meselson, 2005) and microsatellite regions (Campillo et al., 2009). These sequences were widely used to asses stress and physiological status (Kaneko et al., 2002, 2005) or, in the case of 16S, nuclear internal transcribed spacer 1 (ITS1) or *cox1* to study phylogenetic evolution among major taxonomic groups (Fontaneto et al., 2008; Suatoni et al., 2006b;

Gómez et al., 2002). (Molecular biology score: 2). More recently, the complete mitochondrial genome of *Brachionus plicatilis* has been distributed (Suga et al., 2008) as well as a large amounts of other rotifers genetic information (Denekamp et al., 2009; Gladyshev et al., 2008; Welch et al., 2008; Witek et al., 2008; Suga et al., 2007; Pouchkina-Stantcheva and Tunnacliffe, 2005). The complete nuclear genome of *B. plicatilis* is not yet available, despite the fact that the genome of the congeneric species *B. calyciflorus* (Kim et al., 2018) is published *as well as* the transcriptome of *B. koreanus* (Lee et al., 2015) (Genome / Transcriptome score: 1).

Artemia salina is another species commonly used in aquaculture due to the easy of management, culture and hatch as well as the availability of cysts on the international market (Easy availability score: 3). It is characterized by a small size, in particular of its nauplii, (Size score: 2; the size score refers to nauplii and not to adult form due to the fact that adults are not commonly used in research investigations) and by high adaptability at laboratory conditions (Management score: 3). Its physiology as well as reproductive process (Abatzopoulos et al., 2002) and culture techniques are well known and studied (Nunes et al., 2006; Lavens and Sorgeloos, 1984; Brisset et al., 1982). It is possible to culture easily and continuously this species in laboratory (Reproduction score: 3) making the culture process completely automatic (Nash, 1973; Rearing protocols set score: 3).

Artemia sp., also known as “brine shrimp”, has been regarded as a valuable invertebrate model in environmental stress studies related to zooplankton communities thanks to complex adaptive responses evolved by these crustaceans to thrive in hypersaline lakes where various stressors can act, such as lack of food, low oxygen tension, extremely high or low salinity and temperature (Gajardo and Beardmore, 2012). For these reasons it was defined as “the most resistant of all animal life history stages to environmental stress” (Clegg, 2005). Intensive studies were carried out on evolutionary (Yancey et al., 1982) and

ecological (Gajardo and Beardmore, 2012; Tanguay et al., 2004) aspects of the stress response in *Artemia*, with a big focus on the heat shock response and HSPs (heat shock proteins; Feder, 1999), and on the acid-base regulation as well as the ability of different strains or species to manage pH disturbances (Zheng et al., 2015) making *Artemia* a very common model for studies on the physiological and genetic bases of the adaptation (Tanguay et al., 2004). In addition to that, *Artemia* sp. are widely studied for their adaptability and for the production of HSPs as response of stress. Among the various molecules produced by these organisms, the protein p26, a very abundant small-HSP, has the function to establish stress resistance in the developing embryos (Qiu et al., 2007). The activity of this s-HSP is to prevent the irreversible protein denaturation as well as to inhibit apoptotic processes. It has been demonstrated that mammalian cells containing this s-HSP are more thermo-tolerant than the normal ones and are able to limit the heat- and desiccation-induced apoptosis (Wu and MacRae, 2010; Menze and Hand, 2007; apoptosis points: 2).

We used *A. salina* as an alternative model organism to *B. plicatilis* to detect cytotoxic activities in bioassay tests of *H. metazoicum* spent medium. Nevertheless, it has been proven as a model organism easy to manage and culture, its physiology is quite more complicated. Differently from *B. plicatilis*, *A. salina* has physiological adaptations to salt marshes including some resistance ability to bioactive or toxic molecules as demonstrated by comparative studies on aquatic toxicology (Garaventa et al., 2010; Sánchez-Fortún and Barahona, 2005; Varó et al., 2002). Our tests, performed with the abovementioned Cyanobacteria spent medium, demonstrated that *A. salina* is capable to resist, expel or at least partially detoxify bioactive toxic molecules produced by our Cyanobacterium strain of *Halomicronema metazoicum*. Despite the great use of *A. salina* in eco-toxicological scientific research, this species confirms in our test, as well as in Garaventa et al. (2010)

and Nascimento et al. (2000) studies, a wide resistance to various stressors: the biological monitoring and the translation of the observed effects on a more generic biological level, may be unrepresentative and, therefore, misleading (Sasikumar et al., 1995).

Some concerns raised about the responses these organisms are able to give that can be limited by the high adaptability itself due to an evolutionary-developed homeostatic response to stress. The evolution in a so highly variable ecosystems is a great evolutionary constrain and can obscure the effect of climate change in this organism, effect that can be more intense and disruptive in no-highly adaptive species. Additionally, *Artemia* inhabits only extreme environments leading to the consideration that they cannot be considered a representative species for all saline environments. The presence of molecules evolved to stop apoptosis, protein degradation and to assure the survival of embryo to desiccation make these organisms a model to study the process of adaptability to stressful environments but, at the same time, it can be difficult to transfer information obtained by this model to other organisms adding confounding variable to scientific investigation on the effect, for example, of climate change. For these reasons, the suitability of this model for stress response studies should be, at least, questioned. As confirmation of our conclusion on this organism, some authors consider *Artemia* specimens as a “somewhat insensitive organism in ecotoxicological studies” (Nunes et al., 2005; Nascimento et al., 2000). Due to this considerations and abovementioned findings, it is important to underline the need of integrative evaluation of toxicity test performed on high-resistant and adaptable species (Stress responses score: 1).

As regards chemical ecology and infochemicals, *Artemia salina* served, rarely, as a model for zoo-planktonic interactions. Investigations demonstrated that chemical cues produced by predator (kairomones) induce behavioural defence strategies in *Artemia* (Maszczyk and Bartosiewicz, 2012). Charpentier (2017) discovered that kairomones altered photo-

behaviour by increasing visual sensitivity at the photoreceptor level in *Artemia salina*. In addition, it was successfully used to test infochemicals produced by marine fishes inducing diel vertical migrations. Forward and Rittschof (1993) demonstrated that chemicals with a molecular mass less than 500 Da, released from larvae of the Atlantic menhaden (*Brevoortia tyrannus*), a planktivorous fish, are capable to induce and modify phototactic response in *Artemia*. The photo-response was induced using disaccharides with sulfamino or acetylamino group on carbon 2 of a hexosamine.

Despite the fact that simplification is at the base of the model organism theory, *Artemia salina*, as model, has some limitation in the chemical ecology and infochemicals field: a) *Artemia salina* is not representative of zooplankton, and also nauplii cannot be considered true zooplankton. Indeed, *Artemia* lives in shallow, hypersaline, lagoons and ponds; b) *Artemia* lacks of a typical behaviour of zooplankton in the sea. Vertical migration, fundamental in the marine zooplankton behaviour, is reduced in *Artemia* due to the characteristics of its habitat. This means that results obtained cannot be applied, without contextualization, to true marine zooplankton species that live in a very different environment. (Chemical ecology and infochemicals score: 1).

Many genetic tools are available for *Artemia*: inexpensive and high-throughput genomic DNA extraction protocols (Montero-Pau et al., 2008), comparisons of different DNA extraction methods (Xiu and Lu, 2007), studies on expression of the *hunchback* gene (Kontarakis et al., 2006), characterization of polymorphic microsatellite markers (Munoz et al., 2009), a AFLP-Based genetic linkage map for the identification of sex-linked markers (De Vos, 2014; de Vos et al., 2013), studies on the epigenetic response to stress (Norouzitallab et al., 2014) as well as studies on the biodiversity (Boyko et al., 2014; Browne and Bowen, 1991) and phylogenetic relationships among *Artemia* populations (Wang et al., 2008; Baxevanis et al., 2006; Hou et al., 2006) (Molecular biology score: 3).

Mitochondrial genome of *A. tibetiana* (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA207624>), *A. franciscana* (https://www.ncbi.nlm.nih.gov/nucore/NC_001620.1) and *A. urmiana* (https://www.ncbi.nlm.nih.gov/nucore/NC_021382.1) are available. Transcriptome of *A. franciscana* is sequenced and published (Valenzuela-Miranda and Gallardo-Escárate, 2014). In addition, the Laboratory of Aquaculture & Artemia Reference Center of the Ghent University announced that the *Artemia* genome sequencing project is completed leading to the availability of an annotated *Artemia* genome in the next future (not published; <https://www.ugent.be/bw/asae/en/research/aquaculture/research/overview.htm>;
Genome / Transcriptome score: 2).

5.3 MACRO-MODEL ORGANISMS

Hippolyte inermis is a caridean shrimp that lives in *P. oceanica* meadows and in other algae of the Mediterranean basin. It is a quite small (maximum size of males 12 mm and of females 30 mm, Size score: 3; D'Udekem D'Acoz, 1996) protandric consecutive hermaphrodite organism characterized by a complex life cycle: it is characterised by sex reversal that proceeds through a total regression of the male gonad and prosecuting with the development of an ovary from undifferentiated germinal cells. It is a small shrimp, with two reproductive windows in nature (in spring and autumn). The species is part of the *P. oceanica* vagile community and, despite the fact it can live, also, in association with seaweed, the broodstock availability is strictly correlated with the presence, as well as the health status, of seagrass meadows as described by D'Udekem D'Acoz, (1996): "Almost only on seagrasses, but of various species: *Posidonia oceanica*, *Cymodocea nodosa*, *Zostera marina*, *Zostera noltii* (on meadows as well as on isolated plants); very rare on photophile algae" (Easy availability score: 2).

Hippolyte inermis is a seasonal breeder and it has been proven, by our tests, to be an remarkably complex organism that may be difficult to breed and culture at both adult and larval stages (Zupo, 2001; Management score: 1; Reproduction score: 1). We demonstrated that it is important and, contemporarily, possible to simplify the culture of complex organisms and that the optimization of breeding protocols and procedures are a fundamental step in the assess of an organism as model (Rearing protocols set score: 2). Despite the fact that its reproductive process is, in laboratory, a time-spending procedures and that it requires specialized personnel, reproductive processes and development were extensively studied and inquired (Zupo and Buttino, 2001) due to the experiments performed on its unique sex reversal feature and due to the fact that the

apoptogenic compound, produced by the diatom *C. scutellum parva*, is active on this species only during the post-larval periods.

Hippolyte inermis was tested to investigate the effect of acidification on the co-evolutionary relationships with diatoms of the leaf stratum of *P. oceanica* observing the complete miss of the apoptogenic activities on the shrimp androgenic gland. Responses to acidification provided by this species, confirms its importance as a model organism to study stressors and alterations caused by climate changes on marine benthic communities (Zupo et al., 2015, 2016), also thanks to the ability of this species to live in acidified environments (Porzio et al., 2012) (Stress responses score: 2).

Biochemical and molecular processes related to the sex reversal and to the effect of diatom's apoptogenic compound are still to be well understood despite the intensive work profused during the last few decades (Mutalipassi et al., 2018; Maibam et al., 2014; Zupo et al., 2014; Zupo and Maibam, 2010, 2011; Apoptosis score: 3).

The species was proven as a reliable model in chemical ecology experiments (Zupo et al., 2015, 2016; Jüttner et al., 2010), dissimilar and in some cases opposite behaviour responses to VOCs exhibited in our tests, in agreements with the life-cycle stages (adults and juveniles) and to the presence/absence of stressors, in our case acidified test condition and algae cultures. This species is important being the only target for the apoptogenic molecules produced by *C. scutellum* and the bioassay on this organism is necessary to discover the molecular structure, for example by testing diatom HPLC fractions and checking the active ones (Chemical ecology and infochemicals score: 2). In addition, given the peculiar strategy of sex reversal exhibited by *H. inermis* (Zupo et al., 2014; Zupo and Messina, 2007; Zupo, 2000) it is a good model for studies on sex maturation and change, according to environmental or chemical constraints.

A few DNA sequences are provided in literatures for this and phylogenetically related species. Barcode and genetic variability analysis (Terossi et al., 2017; Terossi and Mantelatto, 2012) can be easily performed on *Hippolyte* genus using 16S and Cytochrome Oxidase I, but these are the only genes available (Molecular biology score: 1) and as far as we know there is no genome or transcriptome available for *Hippolyte* species and no transcriptome or genome sequences are available in related genus belonging to the Hippolytidae family (Genome / Transcriptome score: 0; Fig. 5.1).

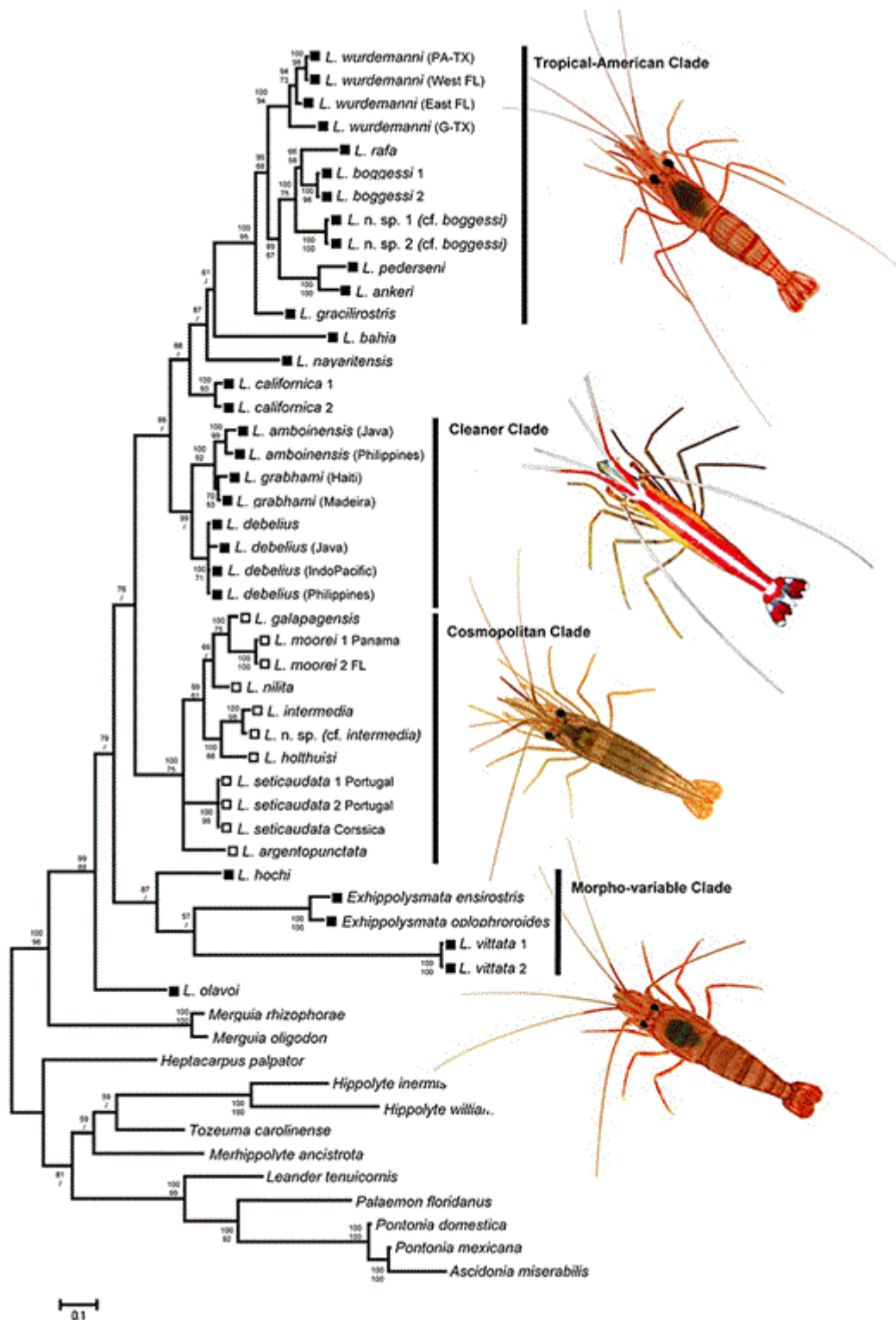


Fig. 5.1: Phylogenetic tree of some selected species of Caridea taxa, obtained from Bayesian inference analysis of the partial 16S rRNA gene (From Baeza, 2010).

Up to date, *H. inermis* is still an unconventional model organism, despite our efforts to optimize culture techniques and to obtain a better understanding of its biological processes. It can be considered the typical “unconventional model organism”, used by a few research groups, with some unique feature and peculiarities that force researchers to use it in very specific research field. The lack of genetic tools, genomes or transcriptomes of species belonging to Hippolytidae family is a limit to the deeper investigation of its physiology. In future, a multidisciplinary approach, exploiting molecular and genetic tools, can achieve the real potential of this model organism. This model, despite the well discussed culture issues, has been proven as an interesting organism to understand the dynamic in *P. oceanica* community.

Idotea balthica is a marine isopod common in seaweed and seagrass of subtidal zone of rocky shores with a wide distribution in nature (Easy availability score: 3). It constitute an exceptionally suitable marine isopod for ecological analysis due to the key role in Baltic sea trophic web, characterized by species-poor ecosystem (Leidenberger et al., 2012). It is a large-enough organism, with a total body size of adults ranging from 1 centimetres in females to 3 centimetres in males (Size score: 3), to allow investigations without the need of sophisticated or expensive techniques (Fava et al., 1992). It is easily adaptable to laboratory conditions showing a short generational turnover and a high reproductive potential. Its culture does not need particular time-consuming technique: at laboratory condition it can be cultured continuously (Strong and Daborn, 1979) and both adults and juvenile, due to their generalist feeder attitude, can be feed with commercial dry food (not showed data; management score: 3).

Despite we observed an easy and continuous reproduction throughout the year at 22 °C and 12/12 h of photoperiod, few old data are available about its reproductive characteristics, incubation periods and embryonic development (Gambardella et al.,

1997; Tuomi et al., 1988; Strong and Daborn, 1979; Mocquard et al., 1978; Howes, 1939) (Reproduction score: 3) as well as we lack of any specific breeding protocols or designed culture system and few data are available about culture methods for marine isopods (Yuh Lee, 1977). The setup of optimized culture and breeding protocols is required in order to limit some issues related to the breeding of this species such as the tendency to cannibalism, particularly observed in overpopulated cultures as well as the inevitable worsening of water quality in close culture systems, due to the high amount of food needed to feed a high metabolic demand of this species (personal observations; rearing protocols set score: 1).

Previous studies suggest that *I. balthica* can be used to investigate adaptation to marginal environments, such as brackish lagoon or estuaries, characterized by continuous unpredictable fluctuations (Fava et al., 1992), and that it can constitute an excellent model for biochemical, physiological and genetic mechanism of the adaptation (Fava et al., 1992; Kaim-Malka et al., 1983, 1990; Tuomi et al., 1988; Saleema, 1986; Bulnheim and Faya, 1982; Mocquard et al., 1978; Bulnheim, 1974) but no studies have been conducted, for example, on the eventual apoptotic effect of anti-grazing molecules produced by micro- and – macro-algae on this organism (Apoptosis score: 1). The stress responses in these species are long-term object of studies and focalized on the respiratory metabolisms (Bulnheim, 1974), on the temperature stress (Roth et al., 2010), on the combined effect of anti-grazing molecules and temperature (Weinberger et al., 2011) or salinity and toxic compounds (Jones, 1973) as well as on the effect of acidification (Wood et al., 2014) and, on *Idotea marginata*, microplastics (Hämer et al., 2014) although some minor concern about the sensibility to stress and the scientific answer given by a so adaptable organism can rise, as discussed above in the case of *Artemia salina* (Stress responses score: 1).

As regards the chemical ecology field, this species is largely studied due to its plant-animal relationship with algae of *Fucus* and *Pilayella* genus, that provide either food and shelters for various *Idotea* species in the Baltic ocean (Orav-Kotta and Kotta, 2004). We tested this model to investigate its response to VOCs produced by two benthic diatoms and a macroalgae cultured and tested at normal pH (8.2) and acidified pH (7.7). As observed in *H. inermis*, adults and juveniles of *I. balthica* showed opposite behavioural responses to infochemicals produced by benthic diatoms and macroalgae. Adults are attracted by macroalgae VOCs, at both culture and test conditions, despite some minor differences. We can speculate that a) the macroalgae *Enteromorpha prolifera* is well adapted to close ecosystems and it does not profoundly alter the own metabolism due to acidified stressor and b) *Idotea balthica* is well adapted to extreme environment too, and its specific chemoreceptors can easily withstand the acidified condition remaining fully functional. On the contrary, juveniles were attracted by *C. scutellum* at normal conditions but did not show any attraction in acidified condition. Despite some limits of the species, such as the high motile activity and the intrinsic ability, especially as regard juveniles, to climb over glass and plastic walls and to squeeze into each cranny of the experimental fields, we can affirm that the species can be considered a good model to understand climate change on a chemical ecology point of view (Chemical ecology and infochemicals score: 2).

A complete transcriptome of *I. balthica* is ready to be published (Keith, unpublished), produced as main topic of a PhD Thesis at the University of Turku with the title: “De novo transcriptome assembly and annotation of the isopod *Idotea balthica*” (Genome / Transcriptome score: 2) but, up to now, only ribosomal and mitochondrial genes are sequenced and available in GenBank (Raupach et al., 2015; Podsiadlowski and

Bartolomaeus, 2006) although extraction protocols are available (Panova et al., 2016; Podsiadlowski and Bartolomaeus, 2006; Wares, 2001; Molecular biology score: 1).

Last but not least, we tested the Mediterranean Sea urchin *Paracentrotus lividus* as a model for ecotoxicology and drug mining studies. The sea urchin *P. lividus* is a common species in aquaculture and fishery (Easy availability score: 3), being an important, widely consumed delicacy present in fish market. In addition, its importance as model organisms raised in the last decades (Faimali et al., 2017) in various fields, such as Evo-Devo research, toxicology and embryology (Ruocco et al., 2017). As model, *P. lividus* despite its medium size (average size 7 centimetres including spines; Size score: 3), it needs well organised and designed facilities (Management score: 2) and studies have been carried out about the feeding in captivity of *P. lividus* adults. Successful maintenance of sea urchins in small-scale closed systems has been described (Cellario and Fenaux, 1990; Fridberger et al., 1979), but such rearing methods are prone to be compromised by overcrowding, low oxygen concentrations, and inadequate water circulation and filtration that can lead to due to cyclic outbreak of bald sea urchin disease. Thus, the culture of *P. lividus* in the laboratory is currently achieved for experimental purposes, culture systems are usually designed for aquaculture purposes (Carboni et al., 2014; Cook and Kelly, 2009; Grosjean et al., 1998) and not on a laboratory scale; however, some small-plant designed for long term culture of *P. lividus* were published (Cirino et al., 2017; Rearing protocols set score: 2).

The reproductive period of this species is variable and related to latitude distribution (de la Uz et al., 2018; Tenuzzo et al., 2012) and for this reason embryos are not available for research purposes all over the year. Reproduction and development of this species are deeply studied (Zupo et al., 2018; Sartori et al., 2016; Fabbrocini and D'Adamo, 2010; Cellario and Fenaux, 1990) due to the fact that embryos are a widely used research tool.

Biggest limit of this species as model, as regard the reproductive feature, is the long time needed to reach the sexual maturity (Grosjean et al., 1998) although some investigation focused on the maintenance of sexual maturation in adults of *P. lividus* using artificial diets (Sartori et al., 2014; Reproduction score: 2).

Paracentrotus lividus affirmed as Mediterranean echinoderm model, thanks to its transparent eggs and embryos as well as its sensitiveness “early development stages of marine invertebrates (fertilization, embryogenesis and larval development) are generally the most sensitive life phases to environmental stresses” (Martin et al., 2011; Moulin et al., 2011). Toxicological test performed on *P. lividus* larvae can be considered nowadays a standard bioassay to find new bioactive molecules or to identify the toxicity or to investigate the effects of already known molecules (Varrella et al., 2016; Privitera et al., 2012; Lopes et al., 2010). Our experiment using *P. lividus* as a model organism was finalized to discover the potential toxicity or antimitotic activity of biomolecules produced by *H. metazoicum*. *Paracentrotus lividus* embryos showed a very high sensibility to cyanobacteria compounds, especially if compared to the other tested organisms. Our bioassays demonstrated a high sensitivity of the model for these kinds of investigations as demonstrated by the concentration threshold within the range of 1:1000 and 1:10000 of the spent medium dilution. Future prospective can be planned, with the design of new bioassays on *P. lividus*, due to the demonstrated sensibility to *H. metazoicum* compounds, using various solvent extraction or HPLC separation of aforementioned Cyanobacterium spent medium (Stress responses score: 3). It has been to be a suitable organism to study autophagy (Chiarelli et al., 2011) and apoptosis: several investigations demonstrated the induction of apoptotic events during embryogenesis of different sea urchin species, in response to different toxic injuries (Agnello and Roccheri, 2010; Agnello et al., 2007). In addition, sea urchin sperms seems to maintain a fully functional apoptotic machinery:

Kazama et al., (2006) demonstrated physiological and morphological changes in the single mitochondrion of the sea urchin sperm, changes similar to those that occur during apoptotic processes. Mitochondrial deformation was observed in sperm of various sea urchin species too: *Hemicentrotus pulcherrimus*, *Pseudocentrotus depressus*, *Mespilia globulus*, *Temnopleurus toreumaticus*, *Clypeaster japonicus*, *Anthocidaris crassispina* (Apoptosis score: 3).

Paracentrotus lividus has been used to investigate the effects of wound activated compounds produced by diatoms. Previous studies demonstrated the relationships between the presence of toxic compounds, e.g. oxylipins, and the production of volatile organic compounds. These compounds, due to coevolutionary relationships, can be recognized by some invertebrates, especially by organisms belonging to the same environment, that are able to detect the presence of activated defences (Maibam et al., 2014; Pohnert et al., 2007; Legrand et al., 2003). Notwithstanding the toxic compounds produced by *H. metazoicum* and tested in our bioassay can be related to secondary metabolites and infochemicals, we still miss the chemical characterization of these compounds and we will not use those data in this section. Despite the fact that test of secondary metabolites can, at least partially, overlap with the ecotoxicological and apoptosis field considered in this thesis, a rich literature is present and *P. lividus* demonstrated to be a good model to verify the effects of these compounds on larvae and adults of a common invertebrate living in various Mediterranean environments (Varrella et al., 2014, 2016; Kâ et al., 2014; Maibam et al., 2014; Maibam, 2012; Marrone et al., 2012; Romano et al., 2010). Regretfully, no data are available about the behavioural response of *P. lividus* larvae to these compounds and this model lack of an interesting aspect of its ecological response to infochemicals (Chemical ecology and infochemicals points: 1).

A huge literature is available about genes and molecular tools for this species, such as RNA extraction protocols (Ruocco et al., 2017), analysis of microsatellite markers (Calderon et al., 2009), studies of population genetic and structure (Maltagliati et al., 2010; Duran et al., 2004), mitochondrial genome (Cantatore et al., 1987, 1989), stress genes (Varrella et al., 2014; Russo et al., 2003) etc. (Molecular biology score: 3).

Although genome is still not available for this species, it is possible to use, at least partially and with appropriate assessments, for example in the primers design, the published genome of the established, phylogenetically related model organism *Strongylocentrotus purpuratus* (Sodergren et al., 2006). On the contrary, a quantitative developmental transcriptome for this species is available and published (Gildor et al., 2015) as well as the mitochondrial genome (Cantatore et al., 1989; Genome / Transcriptome score: 1).

5.4 FINAL REMARKS

In conclusion, selected model organisms have been here ranked according to their unique features and characteristics, as well as the availability of molecular tools and the needs for facility resources. The ranking (Table 5.2), is related to tests and field investigations performed during this PhD course and permits to reach some general conclusions about the features to be taken into account for the choice of the “perfect” model organism, for specific purposes. Evidently, the possibility to approach a model species on a molecular point of view is fundamental for most researches. In fact, we demonstrated that investigations on *C. scutellum*, *H. inermis* and *I. balthica* are limited by the lack of available molecular tools and protocols. Not only molecular and genetic aspects limit these models: the scores obtained are due to some constraints in their culture or due to difficulties (as in the case of *C. scutellum*) in the isolation process. In contrast, *Artemia salina*, a well-established model organism, is easy to culture and it is easily available on the market. This species is provided with several molecular tools that allow in-deep investigations of its physiology. A limit of this species, as well as of *I. balthica*, is its adaptability to extreme environments. As a consequence, the responses of this organism are biased by its adaptability. Thus, the scientific answers provided by tests on this organism should be confirmed by bioassays on other, more sensible, models.

Paracentrotus lividus, *Brachionus plicatilis* and *Artemia salina* reached the maximum scores for most features in our ranking. They showed different features and advantages in experimental researches. *Paracentrotus lividus* is, nowadays, an established model although its genome is not still available and its generation time is not suitable for research needs. It is a key species in several coastal environments and its transparent eggs and larvae, as well as its sensitivity, are fundamental for research investigations. *Brachionus plicatilis* as well, is an important model species for plankton studies and its

sensitivity, as well as the reproductive features and optimized culture techniques, make this species a suitable model for applications in several fields. *Artemia salina* is another small sized model characterized by an easy availability and reproduction, with several rearing protocols available, although the aforementioned issues were raised for applications in ecotoxicology fields.

In light of the results of this Ph.D. project about the appropriateness of various m.o according to their resistance and sensitivity to selected stimuli, it is possible to revise the concept of model organisms and of their use in research. To this end, the final table of scores was submitted to a Correspondence Analysis (Fig. 5.2) and a Cluster Analysis (Fig. 5.3) by means of Statistica 10 (Statsoft Inc., Tulsa, USA).

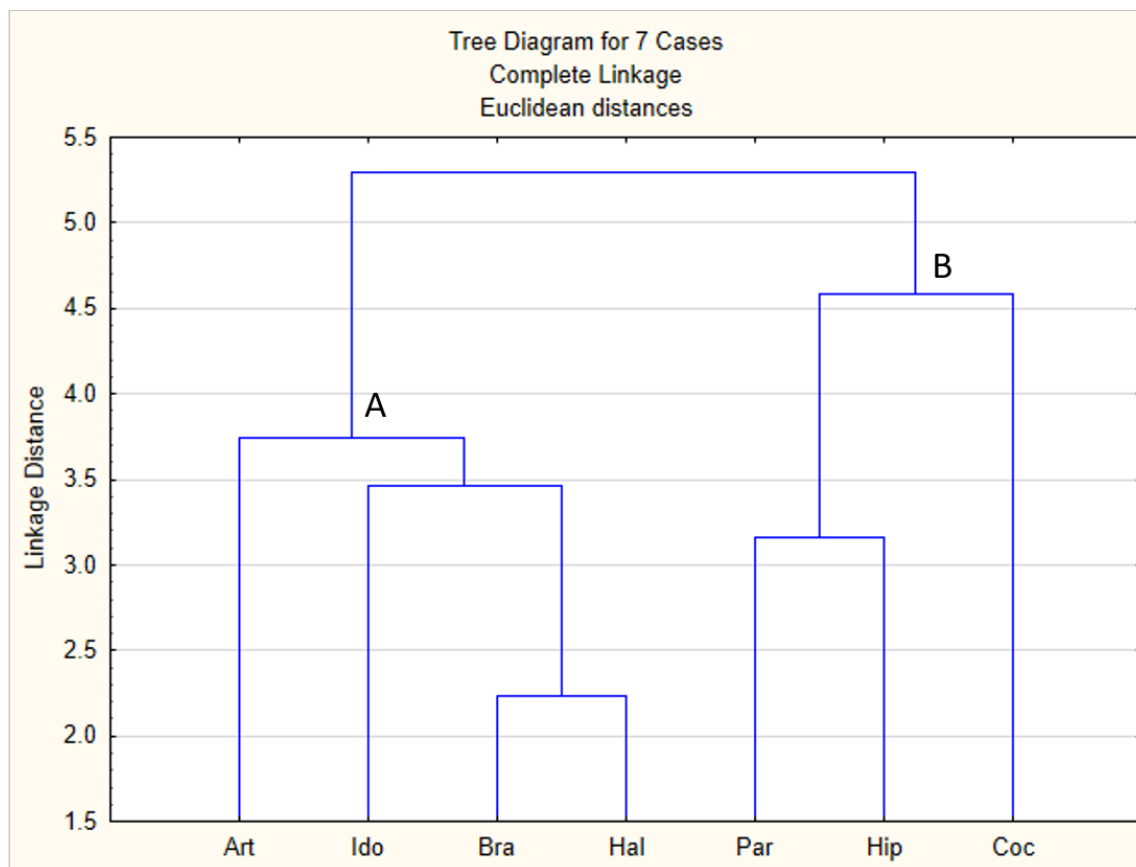


Fig. 5.2: Cluster analyses performed on the ranking matrix of the features of selected model organisms. Codes indicate the selected model species as follow: Art = *Artemia salina*, Ido = *Idotea balthica*, Bra = *Brachionus plicatilis*, Hal = *Halomicronema metazoicum*, Par = *Paracentrotus lividus*, Hip = *Hippolyte inermis*, Coc = *Cocconeis scutellum*

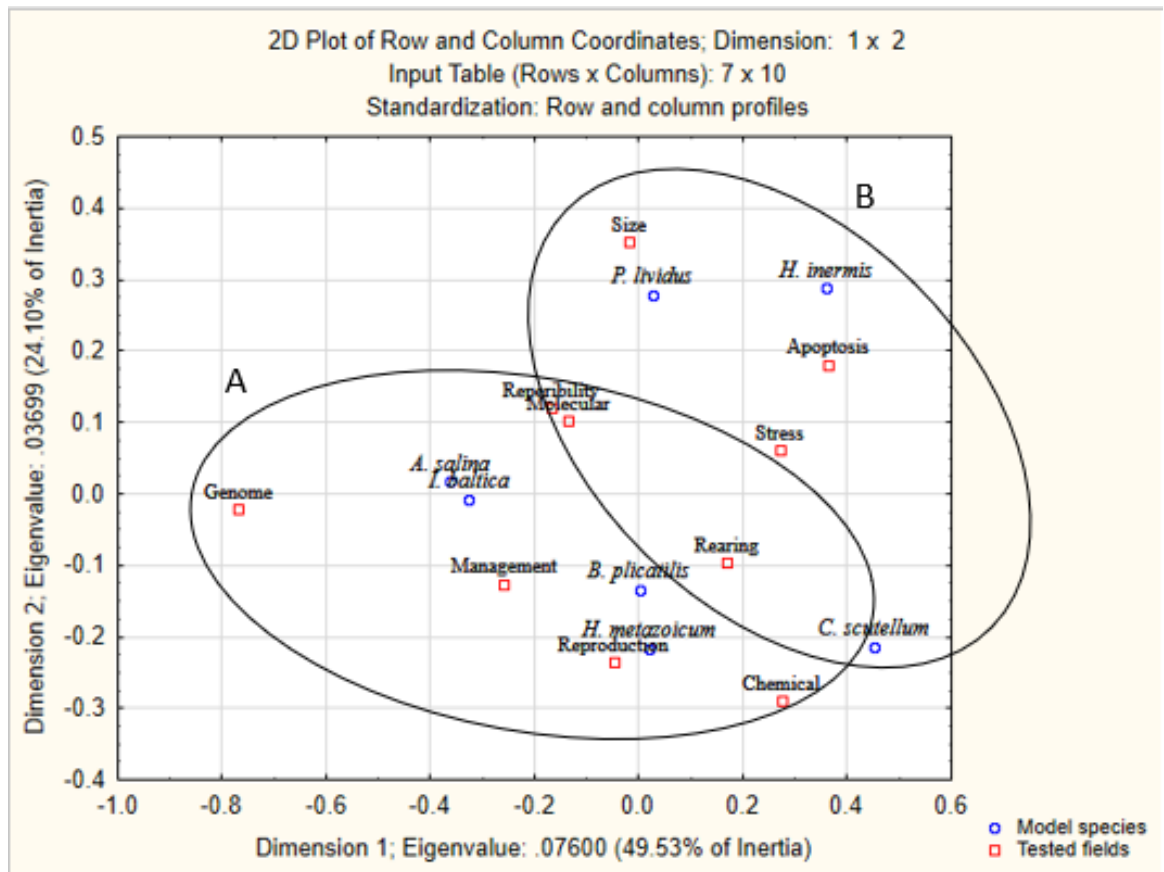


Fig. 5.3: Correspondence analyses performed on the ranking matrix of the features of selected model organisms.

Cluster Analysis indicated the presence of two main clusters among the considered model organisms: the first group (group “A”) contains *Artemia salina*, *Idotea balthica*, *Brachionus plicatilis* and *Halomicronema metazoicum* (Fig. 5.2). The second group (group “B”) is composed of *Cocconeis scutellum*, *Hippolyte inermis* and *Paracentrotus lividus*. Correspondence Analysis (Fig. 5.3) yielded two significant axes expressing, on the whole, the 73.63% of the system total variance, distributed as 49.53 on F1 and 24.10 on F2. The distribution of species along F1 is mainly according to the availability of their genomes/transcriptomes. The distribution of m.o. along F2 is mainly according to their size. This appears consistent with the present trends of research on m.o., since genomic

and molecular analyses represent important fields of studies and, on the other hand, the size of organisms mainly determines their culture techniques and laboratory tools.

Within the group “A”, *H. metazoicum* and *B. plicatilis* are often used in chemical ecology and infochemical studies and they are characterized by high scores obtained in Management, Reproduction and Rearing protocols fields; *Artemia salina* and *Idotea balthica* have a strict relation with the Management field as well as molecular analyses, Genome and Transcriptome and availability of broodstock.

Within group “B”, *Paracentrotus lividus*, *Hippolyte inermis* shows relations with Apoptosis, Size and Stress responses. *Cocconeis scutellum* is close to the group “A”, due to the relation with chemical ecology and infochemicals, reproduction and rearing protocols. However, it also relates to group “B” due to the proximity to Apoptosis and Stress.

In conclusion, we support the hypothesis that, due to the advancement of tools and scientific needs, the concept of m.o. is to be revised and standardized or, at least, we should redefine how to interpret the role and the significance of model organisms in scientific research. Indeed, we can identify two distinct categories of species that internally share common characteristics. On one side (Group A) we identify small organisms, easy to culture and reproduce, supported by advanced molecular tools, to be used as generalist models. They correspond to a classic concept of m.o., indicating a simplified tractable system useful to investigate, as we did in this study, a range of chemical, biological and physical stressors.

In contrast, a second group of species (Group B) is used in research investigations that cannot be strictly defined “model species”. This group is characterized by the lack of such fundamental advantages as maintenance of adults in facilities, reproduction in captivity, easy-to-manage size, strong physiology and easy rearing of larvae. However, they

emerged in scientific research due to fundamental answers they may provide. They have unique and appropriate features, such as specific biochemical or genetic or physiological responses that cannot be investigated using other models. In this view, the use of these “bad models” may conduct to important scientific outputs, especially if we will be able to a) automatize their culture systems (as demonstrated in the frame of this thesis) and b) adapt selected species to breeding conditions. *Hippolyte inermis* represents a key species in our theory. In fact, it demonstrated to be a demanding species in terms of culture and rearing, but it is the only species that enables the detection of the activity of some apoptogenic compounds, thanks to some physiologic peculiarities (as the weak tissue stability of its Androgenic Gland).

Therefore, species like *Hippolyte inermis* cannot be defined, in general terms, as good Model Species but, at the same time, they cannot be excluded by the definition of models, since they are indispensable to explore new aspects of the physiology and test specific hypotheses on basic cellular mechanisms. We can define them as peculiar or “strategic” models. Since the weakness of new, sensitive models is largely influenced by our scarce ability to culture them in continuous, new powerful technologies, as automatic devices based on complex effectors, intelligent controllers and Internet of Things objects, could represent the strategic key to overcome future scientific challenges, transforming “bad models” into smart tools.

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